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Fumonisin B1 toxicity in swine: a comparative analysis of genetically engineered Bt corn and non-Bt corn by using quantitative dietary exposure assessment modeling and ecotoxicological investigations on earthworms

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Fumonisin B_1 toxicity in swine: A comparative analysis of genetically engineered Bt corn and non-Bt corn by using quantitative dietary exposure assessment modeling and ecotoxicological investigations on earthworms

by

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DEDICATION

This is dedicated to all...may we find peace through understanding.

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NOMENCLATURE

ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
Bt	*Bacillus thuringiensis*
BUF	Bt Use fraction
BW	Body Weight
CDF	Cumulative Distribution Function
CP	Crude Protein
DCF	DDGS Concentration Factor
DDGS	Distiller Dried Grains with Solubles
DON	Deoxynivalenol
DUF	DDGS Use Fraction
ECB	European Corn borer
EEC	Estimated Environmental Concentration
ELEM	Equine Leukoencephalomalacia
EWSS	Earth Waste Storage Structures
FB	Fumonisin
FB_1	Fumonisin B_1
FDA	Food and Drug Administration
FICC	Feed Intake Curve Calculator
GE	Genetically Enginnered
LOC	Levels of Concern

ME	Metabolizable Energy
NOAEL	No Observable Adverse Effects Level
NTD’s	Neural Tube Defects
PPE	Porcine Pulmonary Edema
QDEA	Quantitative Dietary Exposure Assessment
QEA	Quantitative Exposure Assessment
Sa/So	Sphinganine to Sphingosine ratio
TCIF	Total Corn Intake Fraction
Total FB	$FB_1+FB_2+FB_3$
USDA	United States Department of Agriculture

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ABSTRACT

Fumonisin B_1 (**FB_1**) is a ubiquitous mycotoxin produced by *Fusarium verticilliodes and F. proliferatum,* and is a common biological contaminant of corn (*Zea mays* L) and other grains. Currently the acute effects from FB_1 exposures are well-documented and managed in the swine industry; however, practices to limit prolonged low-dose exposures to FB_1 have been less fully considered and may negatively impact production efficiency. For decades research involving *Fusarium* and its associated mycotoxins has focused on human, animal and plant health. As result there is limited knowledge of ecological mycotoxicology, with the least understanding pertaining to invertebrate ecotoxicological hazard potential.

Two separate quantitative dietary exposure assessment (**QDEA**) models were conducted to estimate the long-term exposure of FB_1 in nursery and grower-finisher swine diets. Estimated concentrations of FB_1 in swine diets were compared to associated toxicological adverse effects established from the literature. Both QDEA models used deterministic and partially stochastic parameters, which incorporated weekly dietary designs including genetically engineered *Bacillus thuringiensis* (**Bt**)-corn, conventional non-Bt corn, and distillers dried grains with solubles (**DDGS**). Six feeding scenarios differing in the source of corn in diets were modeled to assess variation in FB_1 exposure representing a mixture of (1) Bt and non-Bt grain and DDGS (blended); (2) Bt grain and Bt DDGS; (3) non-Bt grain and non-Bt DDGS; (4) Bt and non-Bt grain; (5) Bt grain; and (6) non-Bt grain.

Nursery phase QDEA long-term exposure estimates (49 d duration) were compared to chronic levels of concern (**LOC**) found in the literature. The initial level of concern (**LOC1**; 1 mg FB_1/kg diet), represents the lowest observed adverse effects concentration resulting in a

decrease of average daily gain in nursery swine. Concentrations of 5 mg FB_1/kg diet represent the second level of concern (**LOC2**), where pulmonary pathological alterations and a significant dose-dependent increase in pulmonary weight may occur in nursery swine. Exposure estimates indicated LOC1 was frequently exceeded regardless of feeding scenario, however; LOC2 was not reached. Diets where the corn fraction was entirely from Bt-corn showed the lowest FB_1 exposure (exceeding LOC1 in 35% of occasions), while either a blended diet or diets using non-Bt grain and DDGS sources more commonly exceeded this threshold (95% of occasions). Based on these estimates, under blended corn source feeding conditions, swine populations in nursery facilities may frequently exhibit incipient effects (i.e., LOC1) of FB_1 toxicity; however, impacts on production efficiency remain uncertain.

Grower-finisher QDEA long-term exposure estimates (20 weeks) were compared to the chronic toxicological incipient LOC (1.0 mg of FB_1/kg of diet). Results from both deterministic and semi-stochastic models demonstrated a distinct difference of FB_1 toxicity in feed between Bt corn and non-Bt corn. Semi-stochastic results predicted the lowest FB_1 exposure for Bt grain with a mean of 1.5 mg FB_1/kg diet and the highest FB_1 exposure for a diet consisting of non-Bt grain and non-Bt DDGS with a mean of 7.87 mg FB_1/kg diet. Results from the deterministic synthesis closely mirrored but tended to slightly under-predict the mean result for the semi-stochastic analysis. This novel comparative QDEA model reveals that diet scenarios where the source of grain is derived from Bt corn presents less potential to induce FB_1 toxicity than diets containing non-Bt corn.

Fumonisins may have the potential for environmental cycling from swine manure agronomic applications and be potentiated further by conservation tillage practices. To assess the ecotoxicological hazard potential of FB_1 on terrestrial invertebrates, an acute 14 day

microcosm study was conducted under controlled laboratory conditions exposing the earthworm species *Eisenia fetida* to FB_1 in an artificial soil (**AS**) system. Exposure concentrations were 1, 3 and 6 fold greater than the estimated environmental concentration (EEC): 2 mg FB_1/kg AS, 6 mg FB_1/kg AS and 12 mg FB_1/kg AS, respectively. Fumonisin B_1 was treated onto alfalfa meal, which was used as the food source. *E. fetida* survival and growth were measured in each treatment. *E. fetida* survival was 100% in all treatments. Results revealed a mean individual percent body weight increase from day 1 to day 14 for the negative control and all FB_1 treatments: 24% (negative control), 34% (1xEEC), 35% (3xEEC) and 30% (6xEEC). Relative to the negative control, treatment 1 increased mean individual percent body weight by 9%, treatment 2 by 10% and treatment 3 by 5%. Mean individual percent body weight declined by 45% for the positive control (500 mg pendimethalin/kg AS). Considering the conservative dosimetry exceeding the EEC by a maximum of six fold, it is concluded that acute environmental exposures of FB_1 presents minimal ecotoxicological hazard potential to *E. fetida*.

CHAPTER 1

GENERAL INTRODUCTION

This dissertation consists of seven chapters: an introduction, two literature reviews, three data chapters and a conclusion. Chapter 1 provides a brief description of the overall dissertation layout, general introduction and rational for the studies conducted. Chapter 2 is a literature review presenting the current state of knowledge with respect to the adverse toxicological effects of fumonisin B_1 (**FB_1**) on the diets of swine. Chapter 3 addresses "Fumonisin B_1 and implications in nursery swine productivity: A quantitative exposure assessment" [1]. Chapter 4 considers "Fumonisin B_1 toxicity in grower-finisher pigs: A comparative analysis of genetically engineered Bt corn and non-Bt corn by using quantitative dietary exposure assessment modeling" [2]. Chapter 5 is a literature review presenting the current state of knowledge with respect to the mycotoxicology pertaining to earthworms. An acute ecotoxicological study of FB_1 exposure to *Eisenia fetida* earthworms was conducted, as described in chapter 6, to assess the ecotoxicological hazard potential of FB_1 on terrestrial invertebrates. Chapter 7 highlights the general conclusions and suggests future directions of the research conducted in this dissertation.

Fumonisin (**FB**), a suite of closely related secondary metabolites is produced by a number of fungal species, although the predominant FB producing fungi are *Fusarium verticillioides* and *Fusarium proliferatum*. Of the existing fumonisins, FB_1 is currently recognized as the most prevalent and toxic form. Fumonisin mycotoxins are ubiquitous in nature and infect corn (*Zea mays L*) and other grains throughout the world. The economic impact as a result of the presence of FB_1 in corn is estimated to result in a market loss of ≈

$40 million from food and feed rejection [3]. The swine production industry is also negatively affected by FB corn contamination, since corn is a major component in swine diet design. Negative FB toxicological effects include porcine pulmonary edema, reduction in average daily gain, hepatic and renal carcinogenesis.

The studies conducted in chapters 3 and 4 are driven by the hypothesis that even though swine diets are managed to limit debilitating acutely toxic levels of FB_1, sustained exposure to low concentrations may negatively impact herd health and production efficiency. Thus the objective was to understand FB_1 exposure characterization during the nursery and grower-finisher production phase. The probability for toxicological adverse effects from diet designs containing differing sources and levels of FB_1 from corn and distillers dried grains with solubles (**DDGS**) was analyzed with a comparative quantitative dietary exposure assessment model using deterministic and partially stochastic parameters, which incorporated weekly dietary designs, Bt use fraction, DDGS use fraction, FB_1 concentration in Bt corn, and FB_1 concentration in non-Bt corn. Effect characterization evaluated published chronic toxicological adverse effects associated with the forecasted FB_1 concentrations relevant to dietary exposure in the nursery and grower-finish production phase. The information synthesized from this investigation will assist in understanding the following areas: (1) determining if diet design can reduce long-term exposure of FB_1 (2) assess the potential exposure impacts of including DDGS in diets (3) providing researchers with a range of estimated environmental concentrations (**EEC**) of FB_1 in various diet designs to aid in dosimetry of chronic studies (4) understanding FB_1 exposure in via diets as a potential FB_1 exposure source in soils from manure applications during agronomic management.

Although the toxicity of FB_1 to mammals and aquatic species is well-described, current FB research describing the environmental fate of FB in soils and the ecotoxicological hazard potential to terrestrial invertebrates is limited. The physicochemical properties of FB such as heat and light stability, high water solubility, low absorption, metabolism, and rapid excretion by animals may result in FB cycling and persistence in the environment. Therefore, chapter 6 is an examination of toxicological effects to terrestrial invertebrates as a result of the potential of FB_1 to concentrate in agricultural soils along a pathway of corn contamination, feed contamination, contaminated manure and exposure to soils due to manure application in agricultural fields as a fertilizer source. Chapter 6 describes an acute ecotoxicological study (14 d) using age-synchronized earthworms (*Eisenia fetida*) to understand the hazard potential from FB_1 concentrations representing the EEC for manure application and using weight gain (variation in body weights) as the toxicological endpoint.

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CHAPTER 2

FUMONISIN MYCOTOXICOSES IN SWINE AND FACTORS INFLUENCING THE OCCURRENCE OF FUMONISIN IN SWINE

Introduction

In 2013, Iowa produced approximately 21.2 million hogs and pigs, accounting for ~31% of the total national production [1]. This large swine enterprise is sustained by the fact that Iowa supplies 16% of the United States corn production [2]. This is particularly important as corn serves as a major dietary component for swine (e.g., ~ 34 to 86% of ration) [3].

Corn may be infected with *Fusarium* fungal species, which are proven to adversely affect swine health and productivity by mycotoxicosis [4]. Some *Fusarium* species generate secondary metabolites known as fumonisin (**FB**). Fumonisin refers to a suite of closely related secondary metabolites produced by various fungal species. *Fusarium verticillioides* (previously known as *F. moniliforme*) and *F. proliferatum* are the most prevalent *Fusarium* fungi producing the more toxic forms of FB (FB_1, FB_2 and FB_3). However recent research has demonstrated that *Aspergillus* section *Nigri* is capable of producing FB_2 [5]. Fumonisin mycotoxins are ubiquitous in nature, infecting corn throughout the world. Corn contaminated by FB has resulted in economic losses involving both corn and swine production. The estimated U.S annual market loss (food and feed rejection) due to FB in corn is approximately $40 million ($14-88 million) [6] and thousands of individual swine have died from FB-induced porcine pulmonary edema (**PPE**) in the United States [7, 8].

Fumonisin pathogenic effects include diseases fatal to both animals and humans, such as equine leukoencephalomalacia (**ELEM**) [9]. Additionally, there is an epidemiologic association of fumonisin B1 (**FB1**) with human esophageal cancer and neural tube defects (**NTDs**) in regions of the world where corn is a major component of diet [10, 11, 12, 13]. In swine, FB causes hepatotoxicity, PPE and cardiovascular adverse effects [7]. Since FB may have both acute and chronic effects, there may be toxicological implications of FB even in the absence of debilitating acutely toxic levels of FB in the diet; therefore, sustained low-dose exposure may negatively impact herd health and production efficiency. Furthermore, there may be human health implications due to the potential occurrence of FB residues in porcine tissues [14, 15]. Ecotoxicological hazard of FB may occur due to the environmental cycling of FB from swine manure agronomic applications and conservation tillage practices.

Early Evidence of Fumonisin Toxicosis in Swine

Documentation of PPE and hydrothorax (i.e., fluid accumulation in the pleural cavity) dates back to 1950 in Hungary, where the anatomical observations were classified as "fattening" or "unique pulmonary edema" [16]. The isolation and chemical characterization of FB in 1988 helped to establish the association between cancer-promoting activity and mycotoxicoses in swine [17, 18, 19, 20]. High levels of corn contamination with *F. moniliforme* mycotoxins in the Southeastern and Midwestern USA (Georgia, Iowa, and Illinois) in 1989 lead to a disease outbreak in swine. The outbreak was identified by extreme acute PPE, abortions, and hepatic damage, resulting in mortality rates in the thousands [7]. Levels of FB_1 in the corn ration screenings from the outbreak ranged from 20 to 330 mg FB_1/kg. Confirmation of FB_1 as the causal agent of the disease was established after 7 days

of exposure to concentrations of FB_1 in feed, similar to that observed during the 1989 outbreak [19, 20].

Mechanisms of Action and Implication to Sustained Health within the Production Cycle

Wang, et al. [21] and Riley, et al. [22, 23] were the first researchers to deduce the principal FB_1 mechanism of action using rat hepatocytes. Results demonstrated accumulation of the sphingoid bases sphinganine and sphingosine, which serve as precursors of sphingolipid biosynthesis. Sphingolipids serve various biological functions as structural maintenance molecules for membranes/lipoproteins and cellular regulators. Different forms of sphingolipids exist due to the long-chain base backbone (sphingoid base). Fumonisin B_1 biochemical mechanism of action incorporates the inhibition of the enzyme sphinganine N-acyltransferase (ceramide synthase), which is responsible for the acylation to sphinganine to dihydrocermide and ceramide. This enzymatic event results in the accumulation of sphinganine. In addition, FB_1 is also capable of increasing the levels of sphingosine by ceramide synthase inhibition, which prevents reacylation of sphingosine from complex sphingolipid turnover. Accumulation of both sphinganine and sphingosine are present during FB_1-mycoxticoses; however, the latter occurs during the late stages of mycotoxicoses (membrane degradation and/or necrosis) [24]. Reduction of enzymatic activity probably results from competitive inhibition due to FB_1 structural similarity to the sphingoid base and fatty acyl-CoA substrates. Depending on the duration of exposure and concentration of FB_1, the magnitude of mycotoxicoses can be reduced or reversed entirely by reduction and/or removal of FB_1-contaminated feed [25]. Reversibility of FB_1- mycotoxicoses is indicative of a mechanism of action involving noncovalent interactions.

Ceramide synthase inhibition takes place in the majority of investigated organisms and is the primary cause of FB_1-toxicity. Toxicity due to sphingoid base accumulation involves a spectrum of negative biological processes; including the inhibition of protein kinase-C activity, the induction of intracellular calcium release, Na^+/K^+ ATP-ase, cellular growth and the activation or inhibition of enzymes responsible for lipid signaling pathways [26, 27, 28]. The above-mentioned adverse effects increase the risk of cytotoxicity, apoptosis and cancer. Fumonisin B_1 exposure studies to LMBc mice have demonstrated an increased frequency of NTDs. Areas where corn is a major dietary component and the occurrence of FB exposure is high (i.e., South Africa, China, Guatemala, and Texas-Mexico border) have displayed increased prevalence of human NTD's and esophageal cancer [11, 13]. Deficiency of folate uptake is hypothesized to induce NTD's resulting from inhibition of sphingolipid biosynthesis [12]. Evaluation of sphingolipid concentrations in serum as a biomarker for the estimation of risk associated with esophageal cancer has not been established with significant association [10]. However, the most sensitive biomarker for the evaluation of FB exposure to swine is the ratio of sphinganine to sphingosine (**Sa/So**) in serum samples [29]. Comprehensive understanding of the full spectrum of FB_1-mycotoxicoses will most likely involve an assortment of biochemical interactions in addition to the Sa/So ratio.

Factors Influencing the Occurrence of Fumonisin in Swine Diets

Factors that affect the occurrence of FB in swine diets comprise three main categories: environmental conditions, agronomic practices and swine management. Environmental conditions include temperature, moisture content, storage conditions, insect damage, age and kernel characteristics of feed as a key component of FB occurrence in swine

diets. It is important to note that the optimum environmental growth conditions for FB accumulation vary between *Fusarium* species. This variability is usually associated with moisture content and the temperature range responsible for optimal growth [30, 31, 32, 33]. In contrast, the other environmental factors (i.e., insect herbivory/stress, plant age and kernel characterizes) usually influence the production of FB regardless of *Fusarium* species. A comprehensive understanding of the environmental factors responsible for FB accumulation remains elusive, with inconsistent findings reported throughout the literature. Elucidation of the mechanisms of action associated with environmental factors and FB production is an essential component for developing methods in reducing FB exposure. See Picot et al. for a review of the overall mechanisms involved in the regulation of FB biosynthesis during corn kernel colonization [34].

It has been determined, in both field evaluations and laboratory experiments, that moisture content and temperature are the most important environmental factors contributing to FB biosynthesis in a large percentage of *Fusarium* species. The effects of moisture content and temperature on the increase of FB_1 in *Fusarium* infected grain has resulted in contradictory findings throughout the scientific literature [33, 35, 36, 37]. The conflicting results reported by researchers reflect the complexity of the interaction between FB production and the environment. Regardless, there is good agreement that moisture content and temperature contribute enormously to the frequency of FB in corn.

Insect Herbivory

There is an inverse relationship between insect damage and field moisture levels during corn production [32]. The larva of the European corn borer (**ECB**) (*Ostrinia nubilalis*) is known to cause kernel and stalk damage in corn. Herbivory damage by the ECB can

provide a route for fungal entry and creates ideal conditions for fungal growth, therefore increasing the probability of *Fusarium* diseases and FB accumulation [38, 39, 40]. Insect herbivory is geographically restricted, with the ECB serving as the major vector of fungal growth on corn in Iowa. Various insects including corn earworms, picnic beetles and western corn rootworm beetles can act as vectors for *Fusarium* species in infected corn [41, 42, 43]. The use of genetically engineered (**GE**) corn designed with resistance toward specific insects (e.g., ECB) and drought resistant hybrids have been shown to decrease FB concentrations in grain during field trials [44, 45, 46, 47]. Further details on the association between insect resistant corn and FB levels will be discussed later in this chapter.

Negative Morphological Alterations in Corn Kernels

Morphological alterations of the corn kernel, also drought related, have been documented to increase FB incidence in corn. Fumonisin accumulation and *Fusarium* kernel rot increase as a result of kernel splitting which is exacerbated as drought conditions worsen [48]. Corn kernels that have thin pericarps are more susceptible to FB exposure, than hybrids that have thick pericarps simply because they are physically less susceptible to insect damage [49].

Growth Stage and Fusarium role

In natural infections, the growth stage of corn plays an important role in the timing of FB biosynthesis. As the corn becomes more senescent, FB biosynthesis appears to increase. Strikingly there appears to be a lack of FB biosynthesis on growing corn tissue, which is characteristic of fungal endophytes (symptomless infections of plant tissue, providing some ecological mutualistic function). In theory most mycotoxins are produced in response to nutrient(s) restriction, which may take place in aging corn. Currently, the symbiotic

relationship that might exist between some *Fusarium* species and corn plants is not well understood [32]. Any development-stage-dependent relationship between FB and corn kernels may involve factors (e.g. signal response, enzyme inhibition, lack of a particular molecule etc.) that regulate FB production. Elucidation of such a factor(s) will aid in understanding how to prevent FB biosynthesis.

Agronomic Management

Limiting the insect herbivory factor (e.g., ECB) has been accomplished with GE corn [44, 45]. Corn expressing Cry δ-endotoxins from the soil bacterium *Bacillus thuringiensis* (**Bt**), some of which are *Lepidoptera* resistant, has reduced ECB damage and, thus, reduced the ability of *Fusarium* to infect corn [50]. Bt-corn became commercially available in the United States in 1995 and since then particular trends have been noted including a lower incidence of *Fusarium* infection and reduction of FB biosynthesis in corn [44, 45]. The lower incidence of *Fusarium* infection presumably reduces the risk of toxic exposure to both livestock and humans. In addition to the reduction in adverse effects in livestock and humans, there are also economic advantages associated with the use of Bt-corn. Strict regulatory policies involving the maximum tolerance levels of mycotoxins for food and feed consumption have been established by several nations. Due to these regulations, market rejection or dramatic price reductions of corn can occur based on the level of mycotoxin contamination [6, 51, 52].

Agronomic management plays an important role in reducing mycotoxin exposure in the feed and food industry, preventing livestock loss and decreased crop prices. Agronomic management decisions are based solely on the options and data available for agriculture producers. Currently the introduction of Bt-corn and drought resistant hybrids (which prevent

kernel splitting result in limiting insect herbivory) have proven successful at decreasing the amount of *Fusarium* and mycotoxin biosynthesis. Clearly, agronomic management decisions can have profound effects on animal (e.g. swine) and human health.

Swine management

Swine and agronomic management coincide with respect to sustaining swine productivity. Decisions about the composition of feed rations and living environments are two of the most significant determinants for successful swine production. The fact that corn constitutes such a high percentage (34 to 86%) of feed must be addressed, due to the elevated risk of FB contamination in corn. Reducing the risk of unfavorable swine health can be accomplished by the type of corn (i.e., Bt-corn, drought tolerant hybrids etc.) used for feed by the swine industry.

Iowa's burgeoning ethanol industry has resulted in corn distillers dried grains with solubles (**DDGS**) becoming an increasingly important energy source in swine rations. Fumonisin is retained and concentrated in DDGS; therefore heightening concern that FB may adversely impact swine production [53]. Thus, consideration of how the use of DDGS may affect long-term exposure to FB in the diet of swine is a further key component for risk assessments [54]. Guidance levels for limiting FB in swine diets have been developed and acute toxicity of FB to swine is not a common occurrence when feed is screened to limit FB.

Effects Characterization

Fumonisin exposure studies have demonstrated that they have the potential to induce a range of acute and chronic mycotoxicoses in a large number of mammals. Oral exposure is

the most prevalent route of FB in terms of swine health and productivity. Fumonisin oral exposure in swine has revealed that although the bioavailability tends to be minimal (3 to 6%); the percentage of toxin that is absorbed is widely distributed throughout the organism (e.g. liver, kidney, large intestine, lung, heart, and brain). Once absorbed, the toxin remains in the organism for an extended period of time due to slow excretion, lack of biotransformation, and enterohepatic recirculation processes [55, 56, 57]. Fumonisin low-dose effects in swine may be further influenced by dietary regulations and modern swine management practices.

The Mycotoxin Committee of the American Association of Veterinary Laboratory Diagnosticians (**AAVLD**) and the U.S Food and Drug Administration (**FDA**) have recommended maximum levels for **total FB** (i.e., FB_1+FB_2+FB_3) in corn and corn by-products for swine (20 mg/kg) and a maximum total FB level in swine rations (10 mg/kg) [58]. Swine management to reduce FB_1 in the diet will limit recurrence of large scale epidemiologic events similar to those recorded in the U.S during 1989-1990 harvest, when FB contaminated corn was responsible for respiratory diseases (i.e., PPE), hydrothorax and hepatotoxicity [7]. Due to the large range in the effects of FB and the possibility for systemic recirculation, swine health and productivity may be affected by chronic low-dose exposures to FB, even when swine management limits acute exposures by AAVLD and FDA recommendations.

Specific Fumonisin Toxicosis in Swine

When discussing the effects of mycotoxins the terms mycoses and mycotoxicoses are sometimes used improperly. Mycoses is the disease state that results from fungal growth on or in an animal host, while mycotoxicoses is any disease caused by exposure to fungal

secondary metabolites (i.e., mycotoxins). Both mycoses and mycotoxicoses cause a broad spectrum of disease states from life-threatening diseases to mild illness. Contaminated feed or food is the major source of FB-mycotoxicoses. Animals experiencing mycoses and/or mycotoxicoses are incapable of transferring any illness to surrounding animals [59].

Effects of mycotoxicoses depend on the same variables one would evaluate when investigating any particular type of xenobiotic or toxin. Variables that play a role in the outcome of mycotoxicoses involve the type and concentration of mycotoxins, duration and route of exposure, sex, health status, age, and possible synergistic or additive effects, diet and species. Summarization of FB-mycotoxicoses in relation to swine can be classified in the following main areas: pulmonary, hepatic and cardiovascular injury.

Porcine Pulmonary Edema (PPE)

Historically PPE is classified as an acute adverse effect associated with FB_1 levels equal to or greater than 10 mg FB_1/kg feed. In order to prevent and treat FB-induced PPE, one must attempt to understand the pathogenesis responsible for such anatomical lung alterations. Currently, the pathogenesis of PPE requires further scientific investigation to elucidate the entire biochemical process of the disease. However, the pathogenesis of PPE may be explained physiologically. Physiologically, PPE can be induced due to alveolar epithelium and pulmonary capillary endothelium damage. Another physiological alteration leading to PPE involves elevated levels of pulmonary capillary hydrostatic pressure due to left-side heart malfunction [8, 60].

Common clinical signs have been observed ~24 hr before swine experience severe pulmonary edema resulting in death. These clinical observations involve lethargy; dog-sitting posture, increased respiratory rates, decreased heart rate, vomiting, diarrhea and excessive

open mouth breathing. Death due to respiratory dysfunction is grossly characterized by fluid in the thoracic cavity and airways, shrinkage and apoptosis of endothelial cells, and increased concentrations of sphingoid bases (i.e., sphingosine and sphinganine) [4, 7, 16, 20, 60, 61].

Investigations involving a 4 week FB_1 dietary exposure study on weaned pigs revealed interesting health implications, resulting in PPE ranging from the classification of mild (10 mg FB_1/kg feed), mild to severe (20 mg FB_1/kg feed), and severe at the highest exposure concentration (40 mg FB_1/kg feed) [62]. These observations suggest that further research is needed to determine safe long-term tolerance levels of FB. In order to provide more insight on the FB tolerance of pigs, weaned piglets were exposed to lower doses (1, 5, 10 mg FB_1/kg feed) for eight weeks. Results from the experiment demonstrated irreversible pulmonary pathological alterations in a dose-dependent manner for all treatment groups, but no obvious clinical signs or physical impairment were observed that are characteristic of PPE. Pulmonary histopathological alterations were classified as proliferation of the connective tissue fibers localized around lymphatic vessels displaying fibrosis, elastosis and/or fibro-elastosis in the alveolar walls [63].

Hepatic Toxicosis

Pharmacokinetic fate and distribution studies with radio-labeled FB_1 indicate that the liver is the organ with the highest accumulation of FB residues, followed by the kidney. High FB exposure in the liver is due to enterohepatic recirculation, resulting in FB reentry to the liver as well as other organs. As a result the liver is a main reservoir for FB_1 accumulation and has a major role in mycotoxin toxicodynamics and toxicokinetics [55, 56].

Hepatic toxicosis is generally localized in the centrolobular and midzonal regions regardless of the route of FB administration [20, 60]. Common FB-mycotoxicoses involves

hepatocellular swelling, vacuolation, cellular dissociation, cord disorganization, fibrosis, necrosis and apoptosis [4, 7, 19, 60, 64]. Clinical chemistry analysis commonly report elevated levels of total bilirubin, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT) alkaline phosphatase (ALP) which are characteristic of hepatotoxicity and disease [4, 7, 20, 60, 62, 64].

Gross pathological observations at low-dose exposures (5 and 10 mg FB_1/kg feed) induced a yellowish color and brittle characteristic to the touch [63]. Although withdrawal of FB after acute or chronic exposure does not guarantee hepatic regeneration [7], the removal of the toxin can result in reversible hepatic effects in cases where injuries were not severe (not attaining the threshold dose for PPE) [20].

Cardiovascular Toxicity

Cardiovascular toxicity has been associated with a decrease in heart rate, contractility, cardiac output, along with increased respiratory rates and mean pulmonary artery pressure [61, 64, 65]. In addition, increased concentrations of Sa/So have been found in plasma and cardiac tissue (right atrium and left ventricle) [61, 64]. The above mentioned adverse effects occurred in acute studies (seven days) in which swine were exposed to rations containing less than 20 mg FB_1/kg body weight and 1 mg FB_1/kg body weight (intravenous exposure for 4 days). Other adverse effects involving cardiovascular toxicity include relaxation of systemic arteries and decreased cardiac contractility. Due to the ability of FB to induce relaxation in smooth muscle tissue, it is regarded as a inotropic and chronotropic agent as a result of its association to decreased heart rates (decreased aortic pulse pressure), which is likely induced by the increased concentrations of sphinganine and sphingosine found in the right atrium and left ventricle [61, 64]. Cardiovascular toxicity induced by FB

can be considered a major contributing factor leading to the subsequent onset of PPE due to increased mean pulmonary arterial pressure. However, mild PPE observed at 10 mg FB_1/kg feed did not demonstrate any cardiovascular toxicity [62]. More in-depth information pertaining to the effects characterization of chronic low-dose exposures (1 to 10 mg FB_1/kg feed) will be reserved for chapters three and four.

Discussion

Considering the acute debilitating effects of FB in swine, attention should be focused on the adverse effects and determination of threshold limits from chronic exposure to low concentrations. Data to address these concerns can be obtained by using quantitative dietary exposure assessment (**QDEA**). Development of a QDEA is based on the concept that risk (the probability for harm to be manifested under realistic conditions) is a function of exposure and effect. Therefore, an assessment of the low-level effects of FB in the diet requires the preliminary evaluation of the major contributing sources of corn for swine consumption, since FB corn contamination is the primary source of exposure. The inclusion of alternative feeds (i.e., DDGS) should also be considered as dietary input parameters. Prevention of insect damage is known to decrease the accumulation of mycotoxins. Therefore, FB exposure characterization should also consider the difference of FB levels in corn transgenically expressing lepidopteran active proteins from *Bacillus thuringiensis*, as compared to non-Bt corn. The presence of Bt-corn may provide feed that contains a higher quality component leading to greater production efficiency in swine through reduction in acute and/or chronic effects associated with FB-mycotoxicoses.

Information on the estimated environmental concentrations (**EEC**) of FB in the diets of swine throughout the production phases (i.e., gestation, nursery and grower-finisher) is limited. This knowledge gap can be bridged through a meta-analysis addressing the concentrations of FB present in corn, and should discriminate between Bt and non-Bt sources as important determinates of exposure [44, 45]. These data would reflect variations in the environmental and genetic factors influencing the occurrence of FB biosynthesis and provide a statistical evaluation of FB production over time. These data in combination with daily corn intake values (percent corn in diet) to achieve recommended dietary energy needs provides a means to calculate the EEC as elaborated in chapters three and four.

Historically researchers have used a Range-Finding approach to assess the thresholds of FB-mycotoxicoses in swine studies. This has resulted in the use of exposure doses that greatly overestimate the concentrations present in the environment (excluding the 1989 FB outbreak). In order to determine an accurate toxicological threshold, no observable adverse effects level (**NOAEL**) and an overall understanding of FB pathogenesis; chronic studies should be designed using the EEC during dosimetry determination. Recently, Delgado and Wolt were the first to forecast the EEC of FB_1 in the diets of nursery and grower-finisher swine and the projection of a chronic toxicological incipient level of concern by assessing FB_1 concentrations in paired trials of Bt and non-Bt hybrids by using QDEA modeling [66, 67]. This information should serve as a framework for estimating FB dietary exposure in swine and is discussed in detail in chapters three and four.

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CHAPTER 3

FUMONISIN B_1 AND IMPLICATIONS IN NURSERY SWINE RODUCTIVITY: A QUANTITATIVE EXPOSURE ASSESSMENT[1]

Abstract

This study estimated the long-term exposure of fumonisin B_1 (FB_1) in nursery swine diets and associated toxicological adverse effects on negative productivity potential using quantitative exposure assessment (QEA). Fumonisin B_1 is a mycotoxin produced by *Fusarium verticilliodes* and *F. proliferatum* and is a common biological contaminant of corn (*Zea mays L*) and other grains. Acute effects from FB_1 exposures are well recognized and managed in the swine industry, but practices to limit prolonged low-dose exposures to FB_1 have been less fully considered and may negatively impact production efficiency. Deterministic (single-point estimates) and stochastic (probabilistic) modeling were performed for comparative analyses of FB_1 exposures originating from genetically engineered *Bacillus thuringiensis* (Bt)-corn, conventional non-Bt corn, and distillers dried grains with solubles (DDGS). Six feeding scenarios differing in the source of corn in diets were modeled to assess variation in FB_1 exposure representing a mixture of (1) Bt and non-Bt grain and DDGS (blended); (2) Bt grain and Bt DDGS; (3) non-Bt grain and non-Bt DDGS; (4) Bt and non-Bt grain; (5) Bt grain; and (6) non-Bt grain. Long-term exposure estimates (49 d duration) were compared to chronic levels of concern (LOC). The first level of concern (LOC1) (1 mg FB_1/kg diet, lowest observed adverse effects concentration) represents a decrease in ADG. Concentrations of 5 mg FB_1/kg diet represent the second level of concern (LOC2), which showed pulmonary pathological alterations and a significant dose-

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dependent increase in pulmonary weight. Estimates indicated LOC1 was frequently exceeded regardless of feeding scenario, but LOC2 was not attained. Diets where the corn fraction was entirely from Bt-corn showed the lowest FB_1 exposure (exceeding LOC1 in 35% of occasions), while either a blended diet or diets using non-Bt grain and DDGS sources more commonly exceeded this threshold (95% of occasions). Based on these estimates, under blended corn source feeding conditions, swine populations in nursery facilities may frequently exhibit incipient effects (i.e., LOC1) of FB_1 toxicity; however, impacts on production efficiency remain uncertain.

Introduction

Fumonisins (**FB**) are a series of mycotoxins ubiquitous in nature, infecting corn (*Zea mays L*) and other grains throughout the world. The majority of FB toxins are derived from *Fusarium verticilliodes* and *F. proliferatum*. The average estimated U.S. annual market loss (food and feed rejection) due to FB in corn is approximately $40 million (Wu, 2006) and thousands of individual swine have died from FB-induced porcine pulmonary edema (**PPE**) in the United States (Haschek et al., 2001).

Recognition of FB acute effects in swine has led to industry measures to protect herd health (e.g., removal, corn channeling to less sensitive livestock, and use of sequestering agents) (Jouany, 2007). Since FB may have both acute and chronic effects, we hypothesize that even in the absence of debilitating acutely toxic levels of FB in the swine diet, sustained exposure to low concentrations may negatively impact herd health and production efficiency. Quantitative exposure assessment (**QEA**) was undertaken to estimate the predominate FB toxin, fumonisin B_1 (**FB1**), in nursery swine diets in order to evaluate possible impact on

swine production. Both deterministic (single-point estimates) and stochastic (probabilistic) analysis were conducted for comparative interpretation of FB_1 exposure originating from genetically engineered *Bacillus thuringiensis* (**Bt**)-corn, conventional non-Bt corn, and distillers dried grains with solubles (**DDGS**). Investigating the associated FB_1 concentrations in both genetically engineered and conventional corn in diets addresses reduced FB_1 concentrations found in Bt-corn (Munkvold et al., 1997).

Materials and Methods

Animal Care and Use Committee approval was not obtained for this study because forecast data were derived from existing literature.

Analytical Model

Information relating to FB_1 exposure and toxicity at prolonged low-doses in nursery swine diets was used for the overall characterization of risk. Our model consists of 3 major components used to characterize the risk of toxicological adverse effects to swine from FB_1 exposure: toxicological effects (levels of concern), swine management, and agronomic management (Figure 1). Six scenarios were developed to consider FB_1 exposure influenced by corn and DDGS as the primary protein source in diets:

- Scenario 1: Blended diet (Bt grain, non-Bt grain, Bt-DDGS and non-Bt DDGS)
- Scenario 2: Bt grain and Bt DDGS
- Scenario 3: non-Bt grain and non-Bt DDGS
- Scenario 4: Bt and non-Bt grain
- Scenario 5: Bt grain
- Scenario 6: non-Bt grain

We conducted both deterministic and stochastic analyses to quantitatively evaluate the conceptual model (Figure 1). Separate sets of worksheets (Microsoft Excel 2007) were used to describe the FB_1 exposure from various diet design scenarios. Deterministic inputs (Table 1) used average, maximum, midpoint or fixed parameter estimates (Cullen and Frey, 1999) and all stochastic modeling (Table 1) used Palisade @Risk 5.0 with random Latin hypercube sampling (McKay et al., 1979; Palisade, 2004).

Each stochastic analysis involved a randomly selected initial seed value for sampling of input distributions and auto iteration to obtain ± 3% mean convergence with a 95% confidence interval. Convergence to this tolerance was typically achieved with < 26,300 iterations. For each stochastic feeding scenario, 7 individual models were created to assess the weekly variation in exposure to FB_1 influenced by differences in diet composition (i.e., corn percentages) throughout the nursery production phase. For any given iteration (i) of the stochastic model, the long-term exposure estimate is the average weekly exposure for the 7-wk long duration (N) of feeding in the nursery (Eq. 1).

$$\text{Exposure} = \frac{\sum_i^N [\text{FB1}]}{N} \quad \text{[Eq. 1]}$$

Common principles for quantitative risk assessment (Vose, 2008) were used to describe risk as the probability of chronic toxicological adverse effects relative to low-dose exposure to FB_1 in nursery swine diets.

Effects Characterization

The following section describes the chronic toxicological adverse effects associated with FB_1 concentrations relevant to dietary exposure in the nursery production phase. Toxicological data provided in this section serves for formulating 2 levels of concern (**LOC**) for FB_1, which characterize the reported toxicological adverse effects.

Rotter et al. (1996) conducted an 8-wk study in nursery swine (6-wk of age, 9 to 12-kg BW). Sixteen Yorkshire barrows and 16 Yorkshire gilts were exposed to fungal cultures in their diet following a 6-d acclimation period. Animals were fed a basal grower diet (15.6% crude protein) formulated to meet NRC (1998) nutrient requirements for swine in mash form containing 50% corn, 25% barley, 18% soybean meal, and 7% additional standard ingredients. Analysis for the presence of FB_1, deoxynivalenol, and zearalenone due to natural contamination of mycotoxin susceptible ingredients showed these ingredients were free of detectable mycotoxins. Water and feed was available ad libitum at all times. Diets contained 0.1 to 10 mg FB_1/kg diet. Each sex consisted of the same treatments, control (0 mg FB_1/kg feed, n = 4), 0.1 mg FB_1/kg feed (n= 4), 1.0 mg FB_1/kg feed (n = 4) and 10 mg FB_1/kg feed (n = 4).

Male pigs fed increasing concentrations of dietary FB_1 showed a linear decrease in ADG (P = 0.059). Significant differences in ADG among different diets were observed throughout the experiment, except for wk 2, 3, 7, and 8. Differences in ADG (kg) for males during the 8-wk exposure for 0 mg FB_1/kg diet = 0.88, 0.1 mg FB_1/kg diet = 0.85, 1.0 mg FB_1/kg diet = 0.81, 10 mg FB_1/kg diet = 0.79. Changes in feed consumption were displayed by males during the first 5 wk of exposures to 0.1 mg FB_1/kg diet. Increased feed consumption was observed by 3, 9, 7 and 5% in weeks 1 to 4 and reduced by 7% in wk 5, respectively, as compared to control animals. Weeks 6 to 8 displayed 6 to 7 % reduction in feed consumption. Investigators reported doses of 1.0 and 10 mg FB_1/kg diet resulted in significant ADG reduction of 8% and 11% in males, respectively, for wks 5 to 8. No statistical difference in feed consumption among varying diets was noted for either sex; however, males fed 10 mg FB_1/kg diet on average ate 10% less than the control animals.

This observation may indicate a palatability issue of concern. Feed consumption of female pigs was slightly above the control diet until wk 4, but no differences in feed consumption or ADG was not observed among diets (Rotter et al., 1996).

Zomborszky-Kovács et al. (2002) conducted a feeding experiment at doses of 1 to 10 mg FB_1/kg diet with weaned barrows (~ 10 kg BW) exposed to FB_1 for 8 wk. Animals were fed a basal diet twice a day according to age, which contained 187 g/kg CP, 12.8 MJ/kg ME and 13.1 g/kg Lys. A 5-d acclimation period was conducted for all treatments before fungal culture was added to diet. Treatments consisted of control (0 mg FB_1/kg feed, n = 4), 1 mg FB_1/kg feed (n= 4), 5 mg FB_1/kg feed (n = 5) and 10 mg FB_1/kg feed (n = 4).

Body weight gains fluctuated throughout the experiment in a toxin dose-dependent manner, but were not statistically significant at the end of experiment. Daily feed consumption was also not statistically significant at termination of experiment. There were, however, dose-dependent chronic changes that were irreversible from FB_1 exposure. Dissection revealed slight lung pathological alterations present in all treatment groups. Pathological alterations in all 3 treatment groups displayed connective tissue fibres, primarily of those around the lymphatic vessels, in the subpleural and interlobular connective tissue of the lungs, extending to the peribronchial and peribronchiolar areas. Authors did not include a scoring/scale criteria for the descriptions of pulmonary pathological alterations. Pathological changes of the lung were found in 1 of 4 animals for treatments with diets containing 1 mg FB_1/kg feed, 2 of 5 animals for treatments containing 5 mg FB_1/kg feed, and 3 of 4 animals for treatments with 10 mg FB_1/kg feed. Exposures of 5 and 10 mg FB_1/kg diet induced a significant ($P < 0.05$) dose-dependent increase in lung weights (g) measured at necropsy

(control 280 ± 56, dose 5 mg FB_1/kg diet = 294 ± 60, and dose 10 mg FB_1/kg diet = 367± 82).

These authors concluded that results from the experiment call attention to the risk of prolonged FB_1 exposure, which has very important public health implications. During the 8-wk dietary exposure to low-dose FB_1 (1 to 10 mg FB_1/kg), observations revealed no clinical signs, significant performance impairment, and no death attributable to toxin exposure, but rendered irreversible the chronic changes (i.e., lung adverse effects) that had already developed in the animals in a dose-dependent manner. Establishment of the lowest observable adverse effects concentration of 1 mg FB_1/kg diet was concluded to be tolerable for efficient swine productivity.

Based on this existing data regarding long-term exposure to FB_1 in nursery swine diets we define, for the purpose of the current QEA, 2 LOC which characterized the reported toxicological adverse effect. The first LOC (**LOC1**) is 1 mg FB_1/kg diet, resulting in 8% significant decrease of ADG when compared to control (Rotter et al., 1996). The second LOC (**LOC2**) of 5 mg FB_1/kg diet demonstrates pulmonary pathological alterations and significant enlargement of the lung in a dose-dependent increase of weight (Zomborszky-Kovács et al., 2002).

Exposure Characterization & Model Parameterization

The subsequent sections detail information necessary to forecast FB_1 exposure and the model parameterization needed to determine risk consistent with the conceptual model (Figure 1). Stochastic parameters consist of specific day in nursery phase, Bt use fraction, DDGS use fraction, fumonisin B_1 concentration in Bt grain, and fumonisin B_1 concentration in non-Bt grain.

Swine Management. We have modeled a typical phase feeding program for swine nurseries consisting of segregated early-weaned (**SEW**), transition, phase 2, and phase 3 (DeRouchey et al., 2007). In order to estimate the dietary concentrations of FB_1 in diet, it was necessary to consider the individual diet development within each phase of the feeding program. Information required for diet development included the following: average duration in nursery, changes in BW over time, ADFI, and total corn intake fraction (**TCIF**).

Diet Design. The diet design used in the model is typical (corn-soybean diet) for swine facilities in the Midwestern USA, as reflected in published guidelines (DeRouchey et al., 2007). Usually, DDGS is used in the late nursery diets. However, for the purpose of this exposure assessment we assume the same DDGS levels in all diets. The following sections discuss the required information for diet development pertaining to swine management.

Specific Day in Nursery Phase. Duration for the nursery phase was based on a population facility size (small: <2,000, medium: 2,000 to 5,000, large: >5,000) as reported by the USDA, Animal and Plant Health Inspection Service (APHIS), which conducted random interviews of swine producers (USDA, 2006). These data provide an average estimate of duration within the nursery of 45.5 d (~ 7 wk). On this basis, the midpoint of 22 d was used as the deterministic value to estimate average BW for nursery swine. For the partially stochastic analysis, the total time of duration in the nursery (7 wk) was uniformly sampled by day for d 1 through 49, with each sampling occasion allowing for an estimation of pig BW based on the specific day in the nursery (Table 2). For each specific day sampled, there is a correlated BW and estimated TCIF in accordance with the Kansas State Growth and Feed Intake Curve Calculator (**FICC**) (see BW and TCIF following).

Body Weight. The FICC was used to determine variation in BW as a function of the specific day during the nursery phase production "R. D. Goodband (Kansas State University, Manhattan, KS, personal communication)". Parameterization inputs for the FICC included initial and eventual finisher close out average BW of 5.67 kg and 120.20 kg, respectively; ADG of 0.39 kg; and the transfer BW from nursery to finishing of 22.68 kg. Body weights generated from the FICC over time were calculated at the endpoints of 7 discrete weekly intervals. Average BW was developed from initial and endpoint FICC nursery BW to represent weekly intervals in the model (Table 2). Body weight information from the FICC generates ADFI and is used indirectly in our model to determine percent corn in the diet.

Total Corn Intake Fraction. The increasing daily feed intake pertaining to percent corn in nursery diets was based on the 4-phase feeding program (DeRouchey et al., 2007) and the FICC. Four-phase feeding programs based on the Kansas State University swine nutritional guide are currently being adopted (Groesbeck et al., 2008). Estimation of the TCIF in diet is based on the BW intervals associated within the 4-phase feeding program (Table 3). Averages determined from maximum and minimum percent corn data were used for SEW and transition diets for the purposes of this analysis; recommended values were used for phase 2 and phase 3 diets.

Agronomic Management

Bt versus non-Bt corn fraction in diet. In order to assess the fraction of Bt and non-Bt corn in swine diets, the number of hectares planted using Bt and non-Bt seed corn was used to estimate corn composition. The USDA, National Agricultural Statistics Service (NASS) estimated in 2008 that 16% of corn planted in the state of Iowa was insect-resistant (Bt) and 53% of all corn planted in Iowa was stacked gene varieties (Bt plus herbicide resistance)

(USDA, 2008). Therefore, in our deterministic model we assume that the TCIF in swine diets has a maximum Bt use fraction (BUF) representing 69% of Iowa corn planted, whereas the stochastic analysis distribution was developed from hectares planted in the major corn production states of the US (Table 4; USDA, 2008). For stochastic analysis Bt-corn adoption fractions were described by the β generalized distribution, because this distribution can represent skewed data (Flynn, 2004). The generalized β distribution has the form:

$$P(x)=\frac{(x-a)^{(p-1)}\times(b-x)^{(q-1)}}{\beta(p,q)\times(b-a)^{(p+q-1)}} \quad \text{[Eq.2]}$$

where *x* is the function of insect-resistant Bt only plus stacked gene varieties; a and b are location parameters (minimum and maximum, respectively) derived from the literature representing the range in Bt-corn fractions and p and q represent shape parameters (Wang, 2005) which were estimated using minimum (α, 0.47), maximum (β, 0.69), mode (c, 0.49), mean (μ, 0.57), *p* (1.02), and *q* (1.23) and as described in the following equations (Table 4).

$$p=\frac{(\mu-a)\times(2c-a-b)}{(c-\mu)\times(b-a)} \quad \text{[Eq. 3]}$$

and

$$q=\frac{(b-\mu)\times p}{(\mu-a)} \quad \text{[Eq. 4]}$$

The β subjective function in @Risk software (Palisade, 2004) was used for the development of the β generalized distribution.

Distillers Dried Grains with Solubles Fraction in Diet. Deterministic modeling used a typical DDGS value reported for nursery swine feeding (i.e., 25% of total diet) and for stochastic analysis a β subjective distribution was generated from the published literature (Shurson et al., 2002; Thaler, 2002; Whitney and Shurson, 2004). Location parameters determined from the literature were 0.05 and 0.25 for minimum and maximum, respectively. Shape parameters are calculated (Eq. [2] and [3]) from mean (0.16) and mode (0.25) values for DDGS as a portion of the diet.

Fumonisin B_1 Concentrations in Bt-hybrids, Non-Bt Hybrids and DDGS. Published data (see Appendix A) describing FB_1 concentrations in paired trials of Bt and non-Bt hybrids were used for estimates of FB_1 in diets, which were expressed as cumulative distribution functions (CDF) describing the empirical data (Figure 2; Munkvold et al., 1997; Munkvold and Hellmich, 2000; Dowd, 2000 Dowd, 2001; Bakan et al., 2002; Magg et al., 2002; Clements et al., 2003; Hammond et al., 2004; Tatli et al., 2004; de la Campa et al., 2005; Papst et al., 2005; Catangui and Berg, 2006). For studies reporting **total FB** (FB_1 + FB_2 + FB_3) in corn, a conversion factor of 1.4142 (R2 = 0.99) was used; developed from the data of Munkvold and Hellmich (2000). Data for total FB were divided by the conversion factor to represent FB_1 on the basis of this relationship. For the deterministic analysis, the arithmetic mean concentration for Bt-corn (2.05 mg FB_1/kg corn), and non-Bt corn (4.15 mg FB_1/kg corn) were used. For the stochastic analysis, the entire CDF was sampled (Figure 2). Estimates of FB_1 concentration in dried (DDGS) used a 3-fold scaling for both deterministic and stochastic analysis as a typically reported value (Wu and Munkvold, 2008).

Results

Existing data were used to forecast long-term FB_1 exposures in feeding scenarios, which may occur in the swine industry. Risk findings were expressed as the probability for exposures to exceed the LOC1 or LOC2 for long-term effects (1 and 5 mg FB_1/kg diet, respectively).

Deterministic Results

All diet scenarios predicted some level of FB_1 exposure exceedance at or above LOC1 (1 mg FB_1/kg diet). However, concentrations exceeding the LOC2 (5 mg FB_1/kg diet) were not demonstrated (Table 5). Diet scenarios where the source of grain or DDGS is derived from non-Bt corn (scenarios 3 and 6) pose the highest probability for exceeding the LOC. Scenarios including only Bt grain (scenario 5) without DDGS exhibited the least mycotoxin exposure. The blended diet design (scenario 1) containing Bt and non-Bt grain and DDGS was ranked intermediate relative to other diet scenarios.

Stochastic Results

Fumonisin B_1 exposures exceeding the LOC1 (1 mg FB_1/kg diet) were observed in all diet scenarios. Variation of FB_1 exposure among scenarios and worst-case incidences representing the 90th percentile of exposure (Table 5) showed the least risk when the diets were developed with Bt grain only (scenario 5). For scenario 5 the LOC1 was exceeded in 40% of occasions, whereas diets composed of the blended regime (scenario 1) and non-Bt and non-Bt DDGS (scenario 3) showed the highest LOC1 exceedance in 95% of cases. The percentile exceedance of LOC1 (1 mg FB_1/kg diet) forecast were:

- Scenario 1: Blended diet (95% of occasions)
- Scenario 2: Bt-grain and Bt DDGS (60% of occasions)

- Scenario 3: non-Bt and non-Bt DDGS (95% of occasions)
- Scenario 4: Bt-grain and non-Bt grain (80% of occasions)
- Scenario 5: Bt grain (40% of occasions)
- Scenario 6: non-Bt grain (85% of occasions)

Diets containing Bt grain (scenario 5) and a blend of Bt & Bt-DDGS (scenario 2) demonstrated potential to decrease FB_1 exposure and, therefore, risk when compared to scenarios containing non-Bt grain and non-Bt DDGS. The mean and median FB_1 exposure estimates were similar, indicative of a normal distribution for the estimated exposures. None of the scenarios investigated demonstrated exposure at or above the LOC2 (5 mg FB_1/kg diet) for worst-case outcomes.

Deterministic versus Stochastic results

The means of stochastic results were lower for scenarios 2, 3, 5 and 6 when compared to the deterministic results (Table 5). The variation relative to FB_1 exposure demonstrates that under the modeling and input parameterization used, most deterministic modeling produced conservative FB_1 exposure estimates relative to the more realistic stochastic estimates. However, the stochastic mean for scenario 1 (blended diet) was approximately 9.7% higher when compared to the deterministic value. Scenario 4 (Bt and non-Bt grain) produced relatively equal mean and deterministic values. Because of variation in input distributions, stochastic results demonstrate that for all scenarios (except scenario 5), exposures above the LOC1 are possible when mean exposure for given population of nursery swine is at or below 1 mg FB_1/kg diet.

Discussion

Stochastic results predicted long-term FB_1 exposures of 1 to 4 mg FB1/kg diet from corn and corn products (i.e., DDGS) in diets of nursery swine. This information may have an influence on diet design and provide background support for chronic low-dose nursery swine toxicological studies.

The blended diet (scenario 1) may represent the industry as a whole in the Midwestern USA based on acceptance of genetically engineered corn (Weber and Richert, 2001; Piva et al., 2001; USDA, 2008; Stein et al., 2009) and the increased use of DDGS in swine diets (Whitney and Shurson, 2004). However, the purchasing practices of individual producers would more likely exhibit only 1 type of corn and 1 type of DDGS for most diets. Under blended feeding conditions swine populations in nursery facilities are predicted to exhibit a high frequency of possible BW reductions characteristic of FB_1 toxicity (i.e., adverse effects associated with LOC1). However, effects representative of PPE are not likely to occur under the given exposures predicted here, since the LOC2 was not exceeded.

Swine management to reduce FB_1 in the diet will limit recurrence of large scale epidemiologic events similar to those recorded in the USA corn harvest during 1989 to 1990 when fumonisin contaminated corn was responsible for respiratory diseases (i.e., PPE) and prenatal/neonatal mortality (Bane et al., 1992; Ross et al., 1990). Management practices relative to fumonisin prevention/reduction strategies in feed include: the transfer or sale of contaminated corn exceeding the FDA guidance levels (> 10 mg/kg, total fumonisin in ration) (FDA, 2001) to less sensitive animals (i.e., bovine), mixing FB-contaminated feed with non-contaminated feed to reduce concentration exposures, proper feed storage

conditions, insect control and the use of adsorbents (i.e., inorganic and organic sequestering agents or binders) (Jouany, 2007).

Since the adoption and release of Bt-corn, its average use in the Midwestern USA has increased from 17% of all corn planted in 2000 to 57% in 2008 (USDA, 2008). As shown by the present analysis, the introduction of Bt-corn in diets has reduced the exposure of FB_1, therefore, limiting exposures below LOC2 (5 mg FB_1/kg diet) for nursery swine. The oral bioavailability of FB_1 in swine is low (~ 3 to 6 %), however, once absorbed the mycotoxin undergoes wide systemic distribution and the absorbed half-life is comparatively long due to enterohepatic recirculation. Swine exposed to chronic diets containing FB_1 may experience accumulation of mycotoxins in the liver and to a lesser extent the kidney (Prelusky et al., 1994; Prelusky et al., 1996). Due to the range of effects that exist and the possibility for systemic recirculation, there remains the likelihood that swine health and productivity may be affected by chronic low-dose FB_1 exposures to a greater extent than predicted here. This is especially true in consideration that low-level exposure to FB will occur from utero-to-finish. Bioaccumulation of FB_1 in nursery swine is not reflected in the source data for the present analysis.

Deterministic versus Stochastic Methodologies

Preference for the use of deterministic or stochastic modeling methodologies is driven by the nature of the concern (e.g., FB_1 low-dose effects) and data availability. Deterministic methodologies serve as a conservative first-step approach in assessing potential risk. In our investigation the deterministic data synthesis demonstrated FB_1 exposure potential commonly occurred at or above the LOC1 (1 mg FB_1/kg diet) for long-term feeding. Several drawbacks are associated with deterministic methods. For example, in our investigation

single-point parameter estimates (e.g., averages, midpoints or fixed values) were used in modeling to describe exposure, which are at times uncertain and may not be representative of true exposures (Cullen and Frey, 1999). Stochastic approaches used representative input distributions to provide a more realistic assessment of exposure (Finley and Paustenbach, 1994; Thompson and Graham, 1996), so that the probable exceedance of LOC for a given nursery population (cohort) could be determined.

Probabilistic Sensitivity Analysis

Sensitivity analysis identifies the input distributions, which most strongly determine output(s) and therefore serves to identify model uncertainties. Multivariate stepwise regression was performed for each feeding interval and scenario. Ranking of sensitivity did not vary among forecasted scenarios and in each instance the concentration of FB_1 associated with non-Bt corn and Bt-corn are the most sensitive inputs to the model. The correlation of FB_1 concentration arising from use of non-Bt corn on exposure estimates is substantially greater than that for Bt-corn (regression coefficients ≥ 0.95 and ≤ 0.30, respectively). For scenarios (5 and 6) containing a single corn source, the associated FB_1 concentrations are the main input influencing exposure.

Uncertainties in Assessment

There are multiple strategies and methodologies to conduct a QEA, each varying in the assumptions and data used. The data used in developing the FB_1 distributions were dominantly (92%) from the Midwestern USA, and while some data were from other regions their inclusion did not alter the observed distribution in FB_1. Environmental conditions (Maiorano et al., 2009) and storage practices influencing FB1 biosynthesis were not included

in our modeling and may be a modifying factor for the actual exposure, which might occur in a given production facility.

Information relating to FB_1 concentration in DDGS is limited. Using the concentration factor of 3-fold for DDGS may over or under estimate mycotoxin exposures when DDGS is used as a corn component. Currently researchers are investigating the mycotoxin concentrations from ethanol production facilities (Wu and Munkvold, 2008), which will improve the ability to predict FB_1 exposure from DDGS in swine diets. The assumption that DDGS was used in all nursery phases of the modeling versus the later phase of nursery production may over estimate FB_1 in scenarios where DDGS is present.

In nature there exist interactions among mycotoxins (e.g., aflatoxin, deoxynivalenol, and T-2 toxin) and their occurrence in grain may impact feed quality in swine production facilities. The toxicological interaction of mycotoxins may be antagonistic, additive, or synergistic depending on the particular mycotoxins considered (Harvey et al., 1995; Dilkin et al., 2003) and were not assessed in our modeling. Creating an aggregated QEA addressing mycotoxin interaction in feed may be relevant to improve the prediction of swine productivity efficiency.

Implications for further research

To the best of our knowledge, this investigation is the first report using quantitative exposure assessment to evaluate nursery swine exposed to low-dose FB_1 in diets. This exposure assessment predicts that there is a high probability that low-level adverse effects (i.e., LOC1) are common-place. This might have broader ramifications regarding FB_1 management, since the current assessment did not investigate the entire production phase (i.e., utero-to-finish). Fumonisin low-dose studies have reported adverse effects in various

target organs (i.e., liver) (Colvin et al., 1993) as well as reproductive effects (Zomborszky-Kovács et al., 2000). It is important to note that the current assessment evaluated nursery swine studies exposed to FB_1 in diets. However, in nature the biosynthesis of FB from *Fusarium* fungi species produces a series of FB toxins (i.e., FB_1, FB_2, and FB_3, ranking in decreasing toxicity, respectively); therefore, our estimates of toxicological adverse effects underestimate the total toxicological burden for FB that may occur in swine diets.

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Table 1. Deterministic (single-point estimate) and stochastic (probabilistic) analysis input assumption for estimating long-term (49 d) exposure to fumonisin B_1 in nursery swine diets[1]

	Deterministic		Stochastic	
Input Parameter	Value	Rationale	Distribution	Parameters[2]
Specific day in nursery phase (D)[3]	22.00	midpoint	Uniform	range: 1 to 49
BW[4], kg	13.04	FICC[3]	BW = *f*(D)	FICC[3]
Bt use fraction (BUF)[5]	0.69	maximum	Generalized Beta	min = 0.47 max = 0.69 mean = 0.57 mode = 0.49 p = 1.02 q = 1.23
DDGS use fraction (DUF)[6]	0.25	maximum	Generalized Beta	min = 0.05 max = 0.25 mean = 0.16 Mode = 0.25 p = 1.20 q = 0.96
Total corn intake fraction (TCIF)[7], kg corn/kg diet	0.52	TCIF=*f*(BW)	TCIF = *f*(BW)	
Fumonisin B_1 concentration in Bt grain ([FB_1]Bt), mg FB_1/kg corn,	2.05	arithmetic mean	empirical CDF[8]	min = 0.01 1% = 0.02 5% = 0.11 10% = 0.14 25% = 0.28 50% = 0.85 75% = 2.69 90% = 5.59 95% = 8.22 99% = 13.43 max = 22.50
Fumonisin B_1 concentration in non-Bt grain ([FB_1]non-Bt), mg FB_1/kg corn	4.15	arithmetic mean	empirical CDF[8]	min = 0.00 1% = 0.05 5% = 0.14 10% = 0.28 25% = 0.78 50% = 2.05 75% = 5.59

			90% = 11.03 95% = 15.91 99% = 28.28 max = 54.45
DDGS concentration factor (DCF)[9]	3.00	fixed	fixed

[1]Fumonisin B_1 exposure equation: TCIF x [FB_1]Bt [(BUF – DUF) + (DUF x DCF)] + TCIF x [FB_1]nonBt {[(1 – BUF) – DUF)] + (DUF x DCF)}.

[2]*p* and *q* = shape parameters

[3]Source: USDA (2006).

[4]Source: Kansas State University Feed Intake Curve Calculator (FICC).

[5]Source: USDA (2008).

[6]Source: Shurson et al., 2002; Thaler, 2002; Whitney Shurson, 2004.

[7]Data modified from the Kansas State University swine nutritional guide (DeRouchey et al., 2007). Starter pig recommendations. Corn was determined by the appropriate TCIF on the basis of BW.

[8]Cumulative distribution function (CDF).

[9]Corn source derived from distiller's dried grains with solubles (DDGS) is estimated to increase fumonisin B_1 concentrations by a magnitude of 3. (FDA 2006).

Table 2. Body weight estimates by 7-wk intervals during nursery phase production as determined from the Kansas State growth and feed intake curve calculator (FICC)[1]

wk	d	BW, kg
1	1 to 7	6.51
2	7 to 14	8.32
3	14 to 21	10.48
4	21 to 28	13.04
5	28 to 35	16.08
6	35 to 42	19.71
7	42 to 49	22.18

[1] R. D. Goodband (Kansas State University, Manhattan, KS, personal communication).

Table 3. Determination of total corn intake fraction (TCIF) in nursery phase diets based on BW[1]

Nursery Phase	Total Corn Intake Fraction (TCIF)[2]
Segregated early weaning: < 5.0, kg BW	0.355
Transition: 5.0 to 6.80, kg BW	0.365
Phase 2: 6.80 to 11.34, kg BW	0.518
Phase 3: 11.34 to 22.68, kg BW	0.522

[1]Data modified from the Kansas State University swine nutritional guide (DeRouchey et al., 2007). Starter pig recommendations. (http://www.ksuswine.org)

[2]Total corn fraction in diet

Table 4. Percentage of insect-resistant *Bacillius thuringiensis* (Bt) and stacked gene varieties (Bt plus herbicide resistance) in U.S. 2008 corn varieties used to estimate Bt use fractions (BUF) in nursery swine diets[1]

State	% Insect-resistant Bt only	% Stacked gene varieties	% Insect-resistant Bt only + % Stacked gene varieties	Fraction of insect-resistant Bt only + Stacked gene varieties
Illinois	13	52	65	0.65
Indiana	7	55	62	0.62
Iowa	16	53	69	0.69
Kansas	25	35	60	0.60
Michigan	15	33	48	0.48
Minnesota	19	40	59	0.59
Missouri	27	22	49	0.49
Nebraska	27	35	62	0.62
North Dakota	24	31	55	0.55
Ohio	12	37	49	0.49
South Dakota	7	58	65	0.65
Texas	20	27	47	0.47
Wisconsin	14	35	49	0.49
			Generalized β parameters[2]	
			Mean = μ	0.57
			Mode = c	0.49
			Max = b	0.69
			Min = a	0.47
			p = α1	1.02
			q = α2	1.23

[1]USDA, National Agriculture Statistics Service (NASS). 2008.
[2]*p* and *q* = shape parameters.

Table 5. Deterministic and stochastic predictions of nursery swine exposure to fumonisin B_1 (FB_1) in diets

Feeding scenarios[1]	Deterministic exposures, mg of FB_1/kg of diet	Stochastic exposures , mg of FB_1/kg of diet		
		Median	Mean	90th
Scenario 1: Blended diet[2]	2.15	2.29	2.38	3.43
Scenario 2: Bt grain & Bt DDGS	1.61	1.21	1.26	2.07
Scenario 3: non-Bt grain & non-Bt DDGS	3.34	2.56	2.63	4.08
Scenario 4: Bt & non-Bt grain	1.43	1.43	1.44	2.05
Scenario 5: Bt grain	1.07	0.90	0.95	1.55
Scenario 6: non-Bt grain	2.23	1.91	1.99	3.05

[1]Corn and corn derived component distiller dried grains with solubles (DDGS) in diet.

[2]Includes a blend of Bt (*Bacillius thuringiensis*) grain, non-Bt grain, Bt DDGS and non-Bt DDGS.

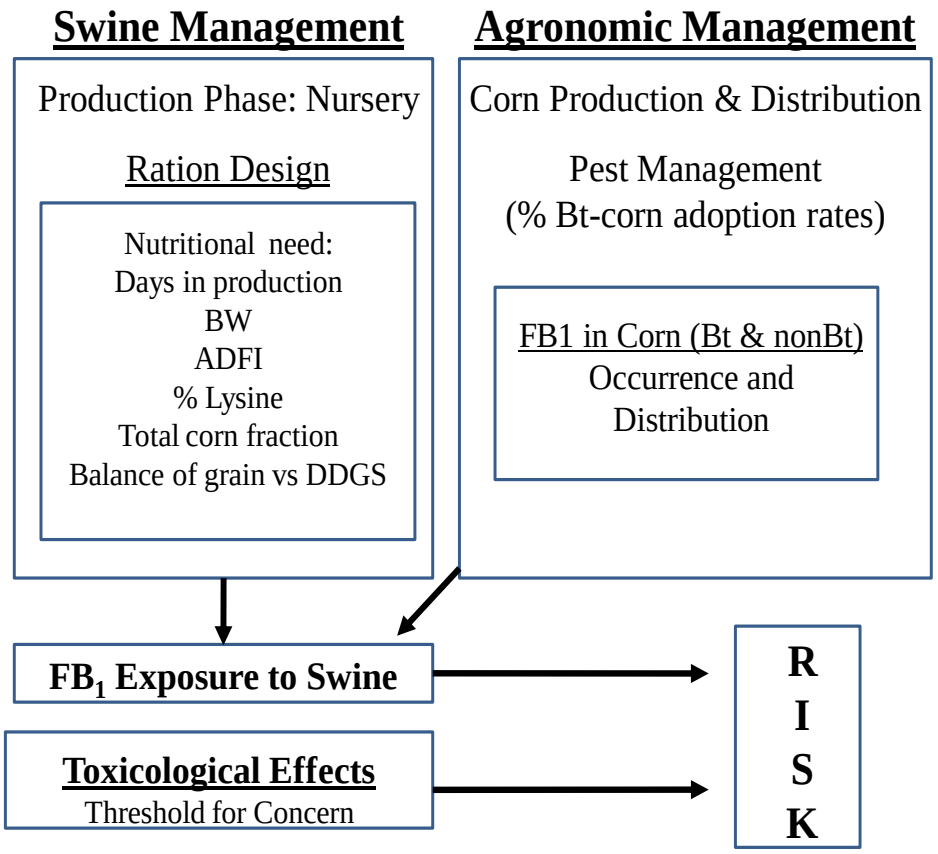

Figure 1. Conceptual model for the characterization of low-dose fumonisin B_1 (FB_1) exposure in nursery swine diets (mg of FB_1/kg of diet) in relation to the chronic toxicological threshold of concern. Risk represents the probability of chronic toxicological adverse effects (i.e., > 1 mg of FB_1/kg of diet) relative to low-dose exposure to fumonisin B_1 in nursery swine diets. Bt = *Bacillius thuringiensis.* DDGS = dried distillers grains with solubles.

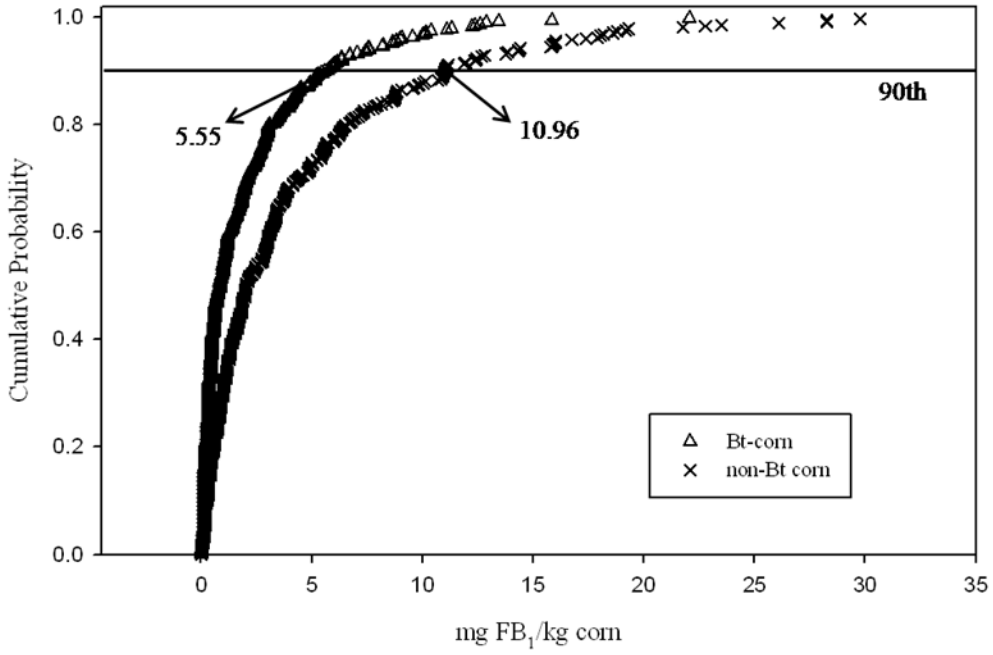

Figure 2. Cumulative distribution of fumonisin B_1 (FB_1) concentrations (mg of FB_1/kg of corn) in Bt (*Bacillius thuringiensis*) versus non-Bt corn; data from 1999 to 2006. Data from Munkvold et al., 1997; Dowd, 2000; 2001; Munkvold and Hellmich, 2000; G. Munkvold, Iowa State University, Ames, unpublished data; Magg et al., 2002; Clements et al., 2003; Hammond et al., 2004; Tatli et al., 2004; de la Campa et al., 2005; Papst et al., 2005; Catangui and Berg, 2006.

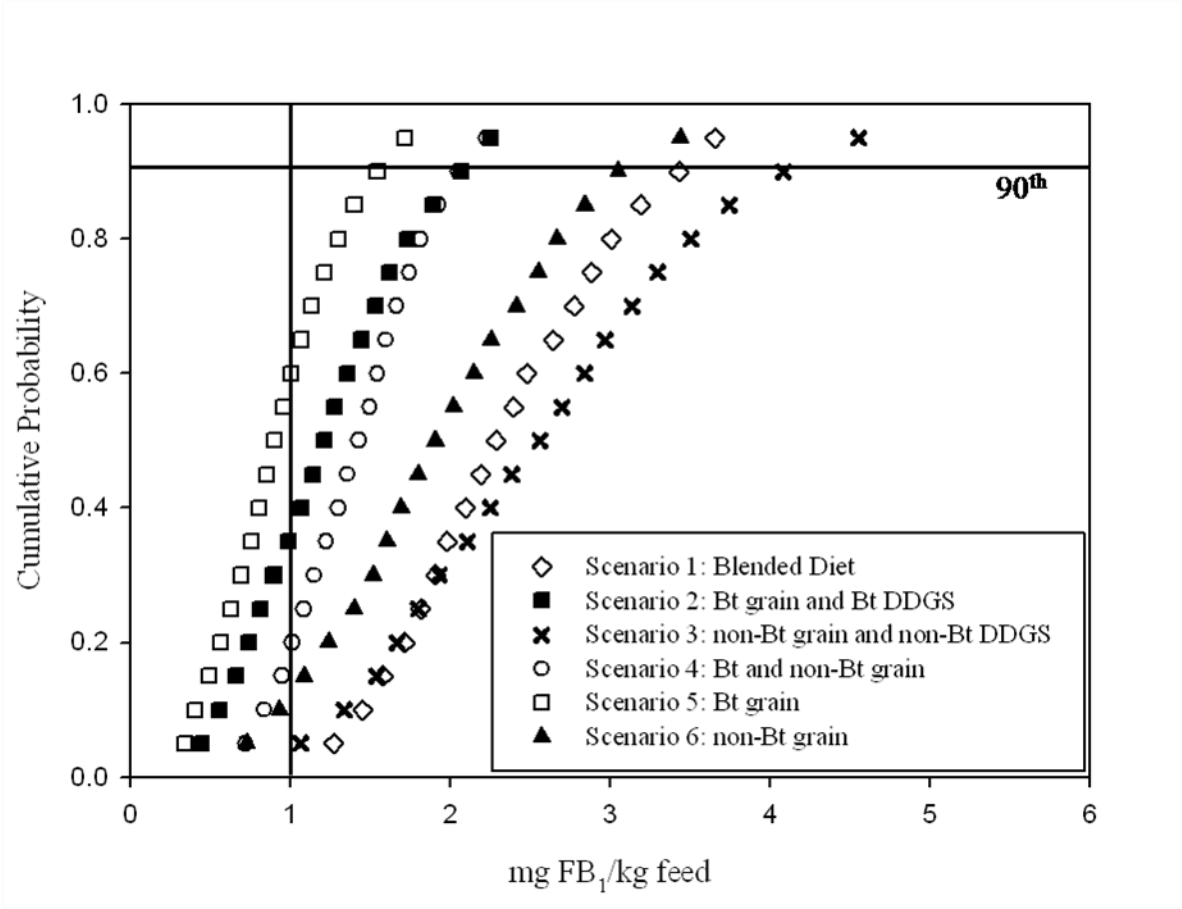

Figure 3. Cumulative distributions of chronic fumonisin B_1 (FB_1) exposure in nursery swine diet scenarios compared to the lower threshold of concern (1 mg of FB_1/kg of diet). Blended diet contains Bt (*Bacillius thuringiensis*) grain, non-Bt grain, Bt DDGS, non-Bt DDGS

CHAPTER 4

FUMONISIN B_1 TOXICITY IN GROWER-FINISHER PIGS: A COMPARATIVE ANALYSIS OF GENETICALLY ENGINEERED BT CORN AND NON-BT CORN BY USING QUANTITATIVE DIETARY EXPOSURE ASSESSMENT MODELLING[1]

Abstract

In this study, we investigate the long-term exposure (20 weeks) to fumonisin B_1 (FB_1) in grower-finisher pigs by conducting a quantitative exposure assessment (QEA). Our analytical approach involved both deterministic and semi-stochastic modeling for dietary comparative analyses of FB_1 exposures originating from genetically engineered *Bacillus thuringiensis* (Bt)-corn, conventional non-Bt corn and distiller's dried grains with solubles (DDGS) derived from Bt and/or non-Bt corn. Results from both deterministic and semi-stochastic demonstrated a distinct difference of FB_1 toxicity in feed between Bt corn and non-Bt corn. Semi-stochastic results predicted the lowest FB1 exposure for Bt grain with a mean of 1.5 mg FB_1/kg diet and the highest FB_1 exposure for a diet consisting of non-Bt grain and non-Bt DDGS with a mean of 7.87 mg FB_1/kg diet; the chronic toxicological incipient level of concern is 1.0 mg of FB_1/kg of diet. Deterministic results closely mirrored but tended to slightly under predict the mean result for the semi-stochastic analysis. This novel comparative QEA model reveals that diet scenarios where the source of grain is derived from Bt corn presents less potential to induce FB_1 toxicity than diets containing non-Bt corn.

[1] James Edward Delgado and Jeffrey D. Wolt. Published in the *International Journal of Environmental Research and Public Health*. (This article belongs to the Special Issue on Environmental Health Risk Assessment). *Int. J. Environ. Res. Public Health* 2011, *8*(8), 3179-3190; doi:10.3390/ijerph8083179

Introduction

Fumonisins are a series of mycotoxins ubiquitous in nature, infecting corn (*Zea mays L*) and other grains throughout the world. Major fumonisin fungi species-mycotoxin associations are derived from *Fusarium verticilliodes* (formerly known as *F. moniliforme*) and *F. proliferatum*. Minor fumonisin sources include *Fusarium nygamai*, *F. napiforme, F. thapsinum, F. anthophilum* and *F. dlamini* [1]. Detection of mycotoxicosis usually involves a close association between the consumption of moldy feed and a specific onset of toxicological effects, altered performance or behavior. Fumonisin-induced porcine pulmonary edema (PPE) is a well-established toxin specific adverse effect [2], and fumonisin also has the potential to negatively impact the food and feed market due to contaminated grain [3].

We recently reported after conducting an exposure assessment that swine populations in nursery facilities may frequently exhibit incipient fumonisin B_1 (FB_1) toxicological effects (i.e., 8% decrease in average daily weight gain) when diets are contaminated at 1 mg of FB_1/kg of diet. The results of Delgado and Wolt [4] have been largely validated by the recent study of Rossi et al.[5] which reports better performance in weaned piglets fed Bt corn compared to piglets fed near isogenic corn and suggests better performance due to lower FB_1 associated with Bt corn [4,5]. The authors' goals in this investigation are to better understand the lifetime exposure (utero-to-finish) and toxicity of FB_1 in pig diets. Due to the variation of percent corn in the diet design throughout the lifetime production, we have divided our QEA modeling into three major components: gestation, nursery, and grower-finisher. This investigation is currently focused on the grower-finisher component and will use our previously established analytical exposure model framework. The only variation in the

grower-finisher model compared to our previous nursery model is the current inputs reflect diet formulation for grower-finisher pigs.

Quantitative exposure assessment (QEA) was conducted using both deterministic (single-point estimates) and stochastic (probabilistic) analysis for comparative interpretation of FB_1 exposure originating from genetically engineered *Bacillus thuringiensis* (Bt)-corn, conventional non-Bt corn and distiller's dried grains with solubles (DDGS). Comparative analysis between Bt corn and non-Bt corn is conducted to determine if FB_1 concentrations differ depending on the corn source, estimating which swine populations may be more susceptible to FB_1 toxicity.

Materials and Methods

Animal Care and Use Committee approval was not obtained for this study because forecast data were derived from existing literature.

Analytical Model

Characterization of risk from FB_1 dietary exposure was estimated by using a conceptual model, which consists of three major components: toxicological effects (levels of concern, LOC), swine management, and agronomic management as described in Delgado and Wolt [4]. Six scenarios were developed to consider FB_1 exposure influenced by corn and DDGS as the primary protein source in diets:

- Scenario 1: Blended diet (Bt grain, non-Bt grain, Bt-DDGS and non-Bt DDGS)
- Scenario 2: Bt grain and Bt DDGS
- Scenario 3: non-Bt grain and non-Bt DDGS
- Scenario 4: Bt and non-Bt grain

- Scenario 5: Bt grain
- Scenario 6: non-Bt grain

Exposure Characterization and Model Parameterization

Information necessary to forecast FB_1 exposure and model parameterization needed to estimate risk consistent with the conceptual model is presented in the following subsections. Each diet scenario required separate sets of worksheets (Microsoft Excel 2010) to describe the FB_1 exposure. Deterministic inputs (Table 1) used average, maximum, midpoint or fixed parameter estimates and all probabilistic modeling (Table 1) used Palisade @Risk 5.7 with random Latin hypercube sampling [6]. The term semi-stochastic will be used to refer to the non-deterministic modeling which does not contain distributions for the inputs of specific week in grower-finisher phase, Bt use fraction in diets and estimations of FB_1 in corn. Refer to Table 1 for descriptions of model input assumptions.

Swine Management. Model parameterization required for diet development included the following: mycotoxin exposure assessed by weekly intervals during the production phase, changes in body weight (BW) over time (i.e., weekly), and total corn intake fraction (TCIF). Information for modeling the diet reflected a typical corn-soybean diet for swine facilities in the Midwestern USA.

Duration of Exposure (Weekly). For the purpose of this dietary exposure assessment, weekly intervals were modeled in order to estimate variations of FB_1 in diets. Estimating exposure by daily intervals was not conducted due to limited changes in diet composition. The sampling of the weekly intervals (i.e., 20 weeks) during production allows for an estimated correlated BW and expected TCIF in accordance with the Kansas State Growth and Feed Intake Curve Calculator (FICC see BW and TCIF below). All deterministic modeling scenarios used the 10^{th} week of production to represent the midpoint of duration.

For the semi-stochastic analysis a total of 20 weekly intervals of production were partitioned into 6 timeframes representative of weight ranges corresponding to the TCIF (Table 2 and Table 3) and sampled by a discrete uniform distribution to estimate the body weight associated with weekly interval.

Bodyweight (BW). Determination of BW was calculated by the Kansas State Growth FICC as a function of the specific week during production [7]. Parameterization inputs for the FICC included initial nursery average BW of 5.67 kg and an average daily gain of 0.39 kg. Initial BW of grower-finisher production was 22.68 kg with an average daily gain of 0.82 kg, and 120.20 kg as the close out average BW. Values of BW were calculated at the end of the indicated week after placement into the grower-finisher phase (Table 2).

Total Corn Intake Fraction. Estimation of the TCIF in diet is based on the BW intervals associated within the 20 week production duration (Table 3) [9].

Agronomic Management

Bt vs. non-Bt Corn Fraction in Diet. Estimation of the fraction of Bt and non-Bt corn in swine diets was conducted by using the percentage of US hectares planting Bt and non-Bt seed corn. The USDA National Agricultural Statistics Service (NASS) estimated in 2010 that 15% of corn planted in the state of Iowa was insect-resistant (Bt) and 61% of all corn planted in Iowa was stacked gene varieties (Bt plus herbicide resistance) [10]. Therefore, in our deterministic model we assume that the TCIF in swine diets has a maximum Bt use fraction (BUF) representing 76% of Iowa corn planted, whereas the stochastic analysis distribution was developed from hectares planted in the major corn production states of the US [10]. For stochastic analysis Bt-corn adoption fractions were estimated by using a beta generalized distribution as described by Delgado and Wolt (Table 4) [4].

DDGS Fraction in Diet. In the Midwestern USA DDGS is increasingly used as an alternative feed source due to increased prices of corn and the widespread availability of DDGS as a by-product of ethanol production. Producers usually design the diets to use the maximum allowed percentage of DDGS. Therefore, DDGS distributions were not used in the models. Both deterministic and semi-stochastic modeling used a maximum of 30% DDGS in the diet formulation, since this value represents acceptable growth performance for swine in the grow-finisher phase [8].

Fumonisin B_1 Concentrations in Bt-hybrids, Non-Bt Hybrids, and DDGS. Paired trials of Bt and non-Bt hybrids were used for estimates of FB_1 in diets, which were expressed as cumulative distribution functions (CDF) describing the empirical data (Figure 1) [11-21]. For specific details pertaining to the CDF calculations, see Delgado and Wolt [4]. Estimates of FB_1 concentration in DDGS used a 3-fold scaling for both deterministic and semi-stochastic analysis as a typically reported value [3].

Information used to generate CDF contains both US and non-US data. We considered very carefully the source data and rationale for inclusion of non-US data sites. Rationale for inclusiveness is to better represent the potential variation in FB_1 due to diverse genetic backgrounds and environments (e.g., location and years). The inclusion of non-US data represents 8.31% (i.e., 32 observations in a total of 385) of the total data used to represent FB_1 in corn (Figure 2).

Effects Characterization

Chronic toxicological adverse effects associated with FB_1 concentrations relevant to dietary exposure in the grower-finisher production phase for formulating the incipient level of concern (LOC) are reviewed in depth by Delgado and Wolt [4] and include the

toxicological study of Rotter et al. [22]. The LOC for this QEA is 1.0 mg of FB_1/kg of diet, which is consistent with the lower LOC used by Delgado and Wolt in the QEA for swine in nurseries [4].

Results

Deterministic Results

Existing data were used to forecast long-term FB_1 exposures in feeding scenarios which may occur in the swine industry. Risk findings were expressed as the probability for exposures to exceed the LOC for long-term effects (1 mg FB_1/kg diet). All diet scenarios predicted some level of FB_1 exposure exceeding the LOC (Table 5). Diet scenarios where the source of grain or DDGS is derived from non-Bt corn (scenarios 3 and 6) pose the greatest opportunity for exceeding the LOC. Scenarios including only Bt grain (scenario 5) without DDGS exhibited the least mycotoxin exposure. The blended diet design (scenario 1) containing Bt and non-Bt grain and DDGS was ranked intermediate relative to other diet scenarios.

Semi-stochastic Results

FB_1 exposures exceeding the LOC were forecasted for all diet scenarios (Figure 3). Variation of FB_1 exposure among scenarios and worst-case incidences representing the 90th percentile of exposure (Table 5) showed the least risk when the diets were developed with Bt grain only (scenario 5) while non-Bt and non-Bt DDGS diets (scenario 3) showed the highest LOC exceedance in 95% of cases. The percentile exceedance of LOC (1 mg FB_1/kg diet) forecasted were:

- Scenario 1: Blended diet (95% of occasions)

- Scenario 2: Bt-grain and Bt DDGS (85% of occasions)
- Scenario 3: non-Bt and non-Bt DDGS (95% of occasions)
- Scenario 4: Bt-grain and non-Bt grain (90% of occasions)
- Scenario 5: Bt grain (70% of occasions)
- Scenario 6: non-Bt grain (95% of occasions)

Discussion

Semi-stochastic results predicted FB_1 ranging from 1.50 to 5.08 and 2.52 to 7.87 mg FB_1/kg diet for the mean and 90th percentile, respectively, where the chronic toxicological incipient level of concern is 1.0 mg of FB_1/kg of diet. Due to the lack of toxicological data in grower-finisher pigs, it is difficult to predict the possible adverse effects induced above the LOC. Additional studies will be required to fully understand the potential negative impact(s) that may be generated from chronic low-dose exposure to FB_1 diets. It is worth noting that the blended diet (scenario 1) may represent the swine industry as a whole; however, it is more likely that diets will contain 1 type of corn source or 1 type of DDGS. Methods of preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds have been discussed by Jouany (2007) [23].

Long-term, low-dose exposures to FB_1 in swine feed (as well as in the diets for other sensitive species with a large component of corn and/or DDGS) may represent a factor limiting health and productivity even when FB_1 is controlled to levels below the acute advisory limits (FDA). Both our previous QEA and the recent study of Rossi et al. show any potential concern for FB_1 chronic toxicity in nursery production will be largely alleviated by the use of Bt corn in the feed [4,5]. In order to understand the lifetime exposure (utero-to-

finish) of FB_1, further QEA models will be required for the gestation phase. This novel Bt and non-Bt comparative dietary QEA model may assist researchers in the dosimetry exposure characterization of experimental designs.

Uncertainties in Assessment

Our current model did not include environmental factors inputs, such as temperature, insect pressure, and storage practice variations [24]. However, since we have used data for FB_1 corn spanning multiple use environments and seven growing seasons, the effects of environmental factors is represented in our sampling distribution.

Estimating the DDGS concentration factor of a 3-fold increase is an overestimate of FB_1 in diets. Preliminary research to determine the DDGS FB_1 concentration factors is estimated to range from 1.5 to 2.8 fold [25]. Inclusion of 30% DDGS throughout the entire grower-finisher production phase has been documented to induce softer pork fat due to high concentrations of linoleic acid in the oil of DDGS, resulting in pork fat iodine that are not acceptable. Therefore, recommendations suggest the removal of DDGS at least 3 weeks before slaughter [8]. The current model included DDGS in diets throughout the production phase without removal.

Acknowledgements

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Table 1. Scenario 1 deterministic (single-point estimate) and semi-stochastic (probabilistic) analysis input assumptions for estimating long-term (20 weeks) exposure to fumonisin B_1 in grower-finisher pig diets [1].

	Deterministic		Semi-stochastic	
Input Parameter	**Value**	**Rationale**	**Distribution**	**Parameters**
Specific Week in Grower-Finisher Phase, (week) [2]	10.00	midpoint	Discrete Uniform	range: 1 to 20
Body Weight [2]**, kg**	79.4	FICC[2]	BW = *f*(D)	FICC[2]
Bt Use Fraction, (BUF) [3]	0.76	maximum	Generalized Beta [4]	min = 0.47 max = 0.69 mean = 0.57 mode = 0.49 *p* = 1.02 *q* = 1.23
DDGS Use Fraction, (DUF) [5]	0.30	maximum	maximum	
Total corn intake fraction (TCIF), kg corn/kg diet [6]	0.820	TCIF=*f*(BW)	TCIF = *f*(BW)	
Fumonisin B_1 concentration in Bt grain, mg FB_1/kg corn, ([FB_1]Bt)	2.05	arithmetic mean	empirical CDF [7]	min = 0.01 1% = 0.02 5% = 0.11 10% = 0.14 25% = 0.28 50% = 0.85 75% = 2.69 90% = 5.59 95% = 8.22 99% = 13.43 max = 22.50
Fumonisin B_1 concentration in non-Bt grain, mg FB_1/kg corn, ([FB_1]non-Bt)	4.15	arithmetic mean	empirical CDF[7]	min = 0.00 1% = 0.05 5% = 0.14 10% = 0.28 25% = 0.78 50% = 2.05 75% = 5.59 90% = 11.03 95% = 15.91 99% = 28.28 max = 54.45
DDGS Concentration	3.00	fixed	fixed	

Factor (DCF) [8]

[1] Fumonisin B_1 exposure equation: TCIF × [FB_1]Bt [(BUF – DUF) + (DUF × DCF)] + TCIF × [FB_1]non Bt {[(1 – BUF) – DUF] + (DUF × DCF)]}. Bt = *Bacillus thuringiensis*. [2] Source: Kansas State University Feed Intake Curve Calculator (FICC). [3] Source: USDA, 2010. Adoption of genetically engineered crops in the US: corn varieties. [4] p and q = beta generalized distribution shape parameters. [5] Source: [8]. [6] Data modified from the Kansas State University swine nutritional guide. Grower-Finishing pig recommendations [9]. Corn was determined by the appropriate TCIF on the basis of body weight. [7] Cumulative distribution function (CDF). [8] Corn source derived from distiller's dried grains with solubles (DDGS) is estimated to increase fumonisin B_1 concentrations by a magnitude of 3.

Table 2. Body weight estimates by weekly intervals during grower-finishing phase production as determined from the Kansas State growth and feed intake curve calculator (FICC) [1] and partitioned timeframes corresponding to total corn intake fraction (TCIF) [2].

Week	Weight, kg	Week	Weight, Kg	Portioned Weekly Timeframes	TCIF[2]
1	27.2	11	85.5	Weeks 1 and 2	0.685
2	32.4	12	91.5	Weeks 3, 4, and 5	0.734
3	37.8	13	97.3	Weeks 6, 7, and 8	0.783
4	43.7	14	103.1	Weeks 9, 10, and 11	0.820
5	49.2	15	108.6	Weeks 12, 13, and 14	0.844
6	55.1	16	113.9	Weeks 15, 16, 17,	0.864
7	61.1	17	118.9	18, 19 and 20	
8	67.2	18	123.7		
9	73.3	19	128.2		
10	79.4	20	132.4		

[1]FICC [7]. [2] Data modified from the Kansas State University swine nutritional guide [9].

Table 3. Determination of total corn intake fraction (TCIF) in grower-finisher pig diets based on bodyweight [1].

Weight Ranges, kg	TCIF
22.7 to 33.6	0.685
34.0 to 54.0	0.734
54.4 to 72.1	0.783
72.6 to 88.0	0.820
88.5 to 104.0	0.844
>104.3	0.864

[1] Data modified from the Kansas State University swine nutritional guide [9].

Table 4. Percentage of insect-resistant *Bacillucs thuringiensis* (Bt) and stacked gene varieties (Bt plus herbicide resistance) in US 2010 corn varieties used to estimate Bt use fractions (BUF) in grower-finisher pig diets [1] [4].

State	% Insect-resistant Bt only	% Stacked genes varities	% Insect-resistant Bt only + % Stacked Gene Varieties	Fraction of insect-resistant Bt only + stacked gene varieties
Illinois	15	52	67	0.67
Indiana	7	56	63	0.63
Iowa	15	61	76	0.76
Kansas	22	40	62	0.62
Michigan	11	44	55	0.55
Minnesota	18	46	64	0.64
Missouri	15	45	60	0.60
Nebraska	22	45	67	0.67
North Dakota	22	37	59	0.59
Ohio	13	36	49	0.49
South Dakota	6	60	66	0.66
Texas	18	40	58	0.58
Wisconsin	13	38	51	0.51
			Generalized β parameters[2]	
			Mean = μ	0.61
			Mode = *c*	0.67
			Maximum = *b*	0.76
			Minimum = *a*	0.49
			p = α1	0.67
			q = α1	0.83

[1] USDA (2010), National Agriculture Statistics Service (NASS). [2] *p* and *q* = shape parameters

Table 5. Deterministic and semi-stochastic predictions of grower-finishing pig exposure to fumonisin B_1 (FB_1) in diets.

Feeding Scenarios[1]	Deterministic exposures mg FB_1/kg diet	Semi-stochastic exposures mg of FB_1/kg of diet		
		Median	Mean	90th
Scenario 1: Blended Diet[2]	2.86	3.46	3.50	5.08
Scenario 2: Bt grain & Bt DDGS	2.32	2.25	2.40	4.01
Scenario 3: non-Bt grain & non-Bt DDGS	4.69	4.88	5.08	7.87
Scenario 4: Bt & non-Bt grain	2.09	2.13	2.19	3.20
Scenario 5: Bt grain	1.68	1.43	1.50	2.52
Scenario 6: non-Bt grain	3.40	3.02	3.11	4.97

[1] Corn and corn derived component distiller dried grains with solubles (DDGS) in diet. [2] Includes a blend of Bt grain, non-Bt grain, Bt DDGS and non-Bt DDGS.

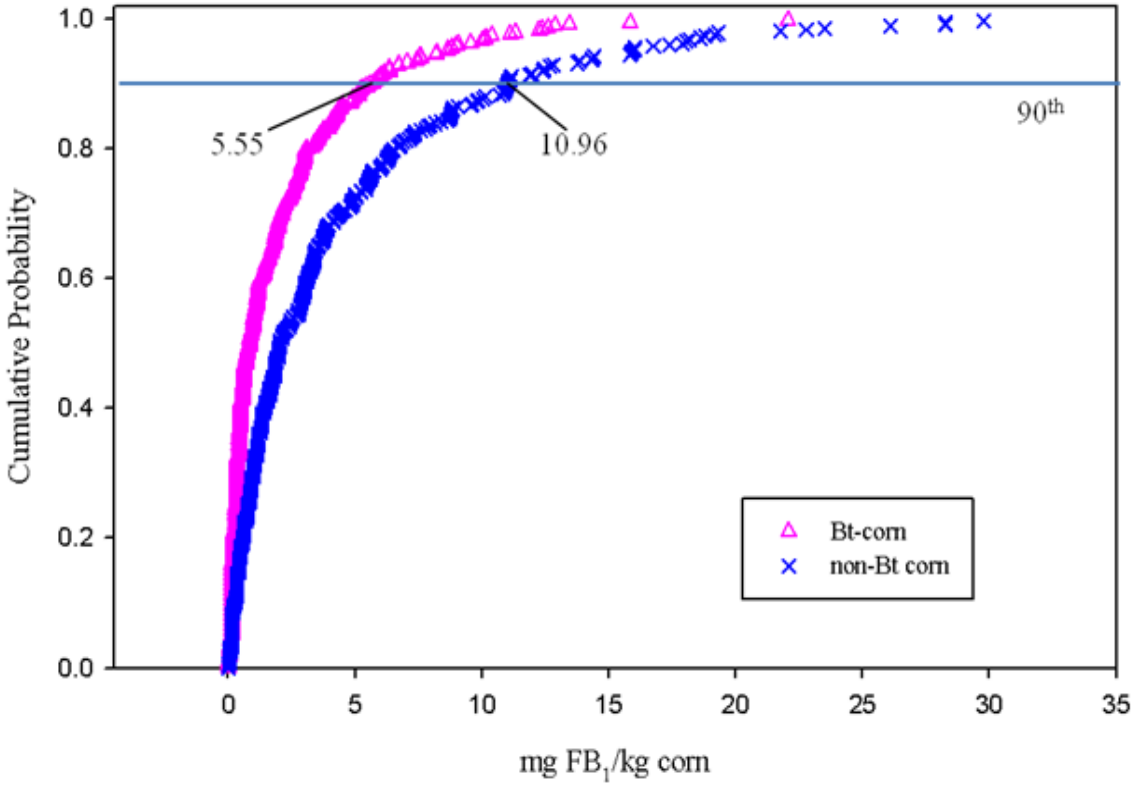

Figure 1. Cumulative distribution of fumonisin B_1 (FB_1) concentrations (mg of FB_1/kg corn) in Bt (*Bacillius thuringiensis*) vs. non-Bt corn; data from 1999 to 2006 [11-21] from Delgado and Wolt [4].

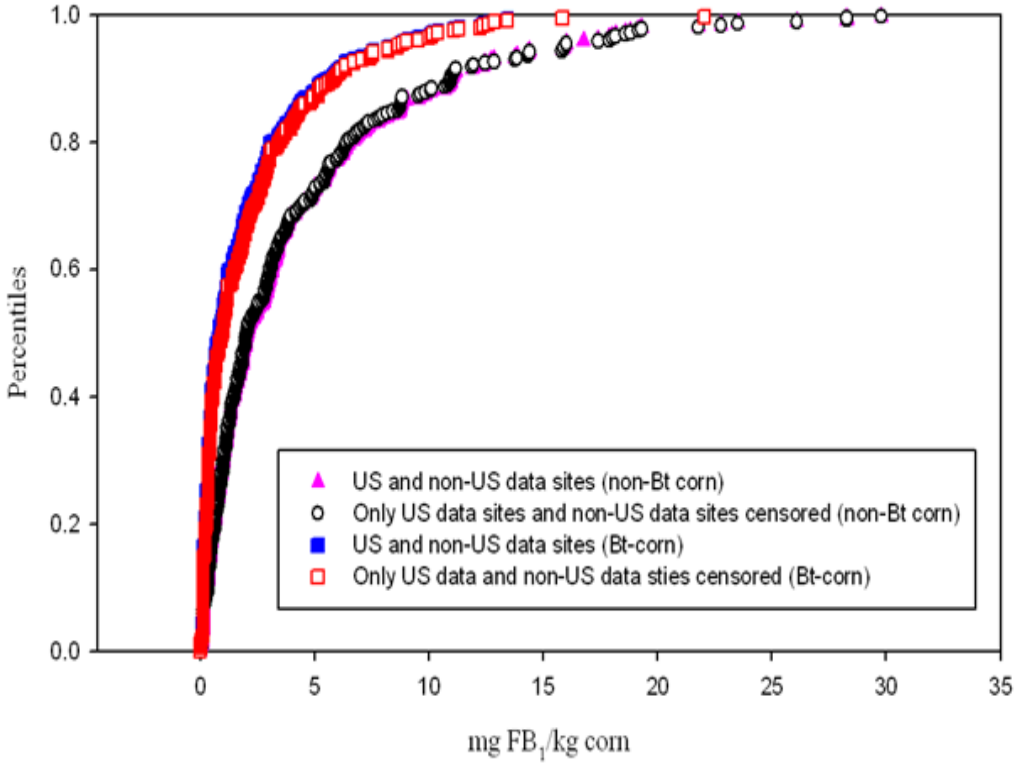

Figure 2. Comparison of US and non-US data versus censoring non-US data showing a cumulative distribution of fumonisin B_1 (FB_1) concentrations (mg of FB_1/kg corn).

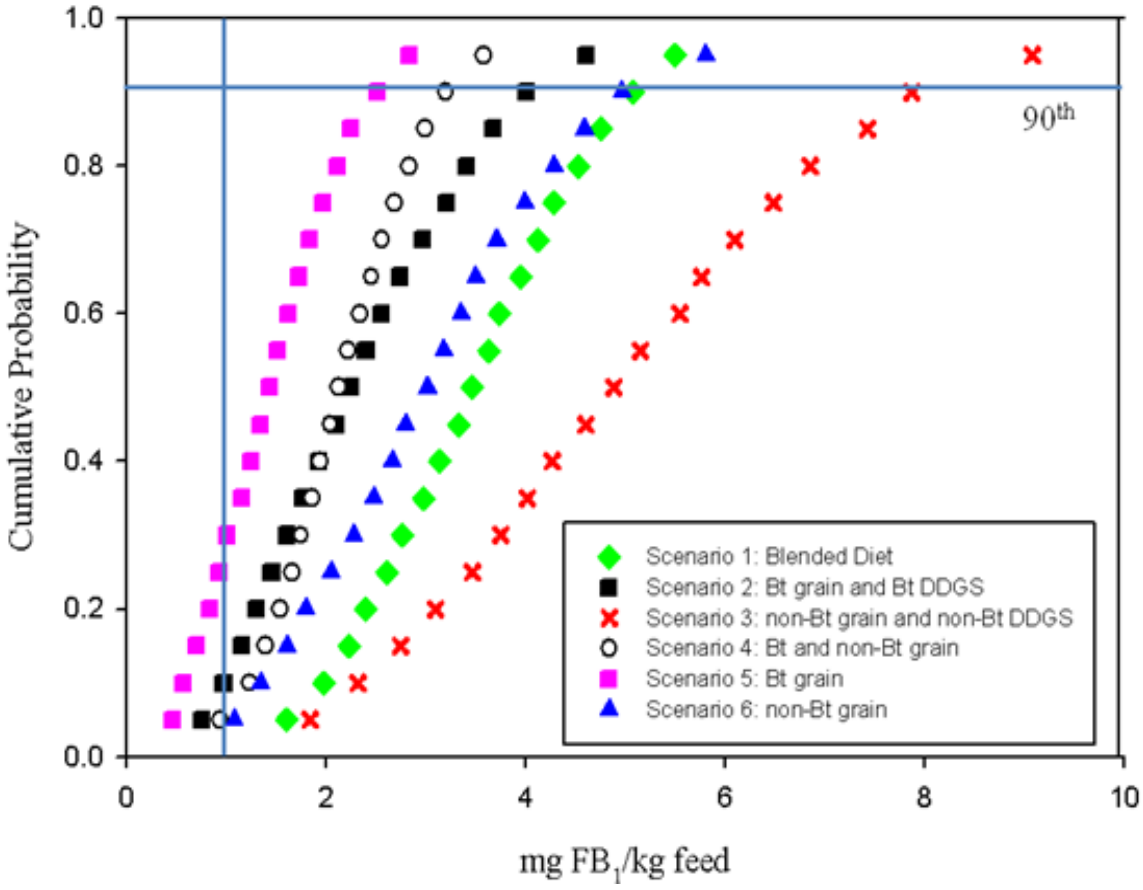

Figure 3. Cumulative distributions of chronic fumonisin B_1 (FB_1) exposure in grower-finisher pig diet scenarios compared to the lower threshold of concern (1 mg FB_1/kg diet). Blended diet contains Bt grain, non-Bt grain, Bt DDGS, non-Bt DDGS.

CHAPTER 5

EFFECTS CHARACTERIZATION OF *FUSARIUM* AND MYCOTOXIN EXPOSURE TO EARTHWORMS: A REVIEW

Introduction

Mycotoxins have been defined as 'natural products produced by fungi that evoke a toxic response when introduced in concentrations to higher vertebrates and other animals by natural route(s)' [1]. The first mycotoxicosis observations in humans date back to the Middle Ages when disease outbreaks with gangrenous symptoms known as Saint Anthony's Fire were documented [2]. Since that time mycotoxicology research has focused on mycotoxins produced by plant diseases and on human and animal exposure to plant-based food or feed products. However, research addressing the potential ecotoxicological hazard of mycotoxins is limited. Some mycotoxins are suspected to have the potential for environmental cycling, which is facilitated by common agronomic practices like swine manure applications, and research has shown an increased prevalence of *Fusarium* species, which can produce mycotoxins, in soil where conservation tillage is practiced [3, 4, 5, 6]. The *Fusarium* fungi species are capable of producing different mycotoxins. *Fusarium graminearum* and *Fusarium culmorum* are known to produce the mycotoxin deoxynivalenol (**DON**), also known as vomitoxin. Deoxynivalenol is most prevalent on barley, corn, rye, and wheat, and if consumed has the potential to cause mycotoxicosis in a both animals and humans. While researchers have recently made advances in understanding aquatic mycotoxicology and its ecotoxicological effects on aquatic life [7, 8, 9], the ecotoxicological effects of mycotoxins

on terrestrial invertebrates are largely unknown. With respect to earthworms studies they are limited to *Fusarium*-infected wheat [10, 11, 12, 13].

A Review of Ecotoxicological Studies

Oldenburg et al. (2008) conducted a microcosm study in which three toxigenic strains of *Fusarium culmorum* (F.c. 34, F.c 35 and F.c 36) were used to artificially infect wheat straw and placed on the soil surface with *Lumbricus terrestris* earthworms [10]. Percent soil surface cover by wheat straw, body weights, *Fusarium* biomass, and DON removal were recorded at 5 and 11 weeks for both infected and uninfected straw. *Fusarium* biomass and DON removal were also recorded in the absence of *L. terrestris* to determine any differences in percent removal caused by the presence of *L. terrestris*. At week 5, the straw surface removal percentages were 56% for the infected straw with *L. terrestris*, 38% for uninfected straw with *L. terrestris*. Body weight of *L. terrestris* recorded during week 5 showed an increased weight for both infected straw (6.5%) and uninfected straw (1.4%). *Fusarium* biomass removal in the microcosms without *L. terrestris* was 85%, and no *Fusarium* biomass was detected in the microcosms containing *L. terrestris* at week 5. The recorded initial concentration of DON on infected-straw on the soil surface was 146.7 ± 18.1 mg DON/kg soil. The DON concentration was reduced by approximately 77% in microcosms without *L. terrestris* and by 99.7% in microcosms with *L. terrestris* at week 5. At 11 weeks, the straw surface removal percentages were 74% (infected straw) and 53% (uninfected straw). By week 11 a body weight decrease was shown for *L. terrestris* in both straw types, though the reduction was less for infected straw (8%) than for the uninfected straw (14%). *Fusarium* biomass removal was nearly complete in the microcosm without *L. terrestris* at week 11 and was already undetectable in the microcosm with *L. terrestris* at week 5. At week 11, DON

concentration was reduced by 88% in microcosms without *L. terrestris* and by 99.9% in microcosms with *L. terrestris*. They concluded *L. terrestris* have a feeding preference for *Fusarium*-infected straw and that consumption of DON did not reduce body weights or reduce feeding behavior [10]. The consumption of *Fusarium*-infected straw and/or DON induced weight gain for the initial part of the study (5 weeks) and later resulted in decreased weight gain at the end of the study (11 weeks), which was most likely caused by the lack of food.

The uptake and bioavailability of DON was later addressed by the same research group (Schrader et al., 2009) [11]. Schrader et al. (2009) used the same experimental design of Oldendburg et al. (2008) [10, 11]. Body weight of *L. terrestris* recorded during week 5 showed an increased weight for infected straw (6.5%) and no distinct change was observed for uninfected straw. Week 11 showed a body weight decrease for both straw types, though the reduction was less for infected straw (8%) than for the uninfected straw (14%). The initial concentration of DON on infected straw was 146,660 µg/kg soil. In the presence of *L. terrestris*, DON was recovered from soil at a concentration of 430 and 175 µg/kg soil for weeks 5 and 11. Earthworm gut tissue, gut content and body wall were examined for the presence of DON. Within the week 5 study DON was only detectable in gut tissue (200 µg/kg); however, for week 11 DON was detected in gut tissue (272 µg/kg), body wall (79 µg/kg) and gut content (51 µg/kg) with significant differences among each location ($P<0.001$).

Wolfarth et al. (2011) conducted a study similar to that described above; however, they used a different earthworm species (*Aporrectodea caliginosa)* and only collected data on week 5 [12]. *A. caliginosa* is defined as an endogeic species, with horizontal burrowing

behavior and feeding that usually takes place in the upper part of the soil surface. Results showed all *A. caliginosa* survived the study. Although the body weight of *A. caliginosa* increased for both infected straw (18%) and uninfected straw (9%), the difference in body weights between infected and uninfected treatments was not significant. The initial concentration of DON on infected straw aboveground was 123,070 ± 5,886 µg/kg soil. The removal of DON with and without the presence of *A. caliginosa* was approximately the same (70%). *Fusarium* biomass aboveground removal for infected straw was approximately the same (50%) for microcosms with and without *A. caliginosa.* Overall, results from the study showed that *A. caliginosa* has negligible contributions to the removal of *Fusarium* biomass on the top surface of soils. It is more likely that removal of *Fusarium* biomass will take place in the shallow subsurface of soils in environments containing earthworms of the *Aporrectodea* genus.

To better understand *L. terrestris* and *A. caliginosa* decomposing ability of *Fusarium* biomass and DON, Wolfarth et al. (2011) conducted similar experiments as described above but in field conditions [13]. Mesocosms were constructed on the topsoil of fields where conservation tillage was practiced. Treatments within the mesocosms contained both earthworm species, species separated, and without earthworms. The mesocosms contained both *Fusarium*-infected wheat straw contaminated with DON (123,070 µg/kg soil) and uninfected wheat straw. The difference in earthworm biomass was not statistically significant between treatments of infected versus non-infected straw ($F = 0.179$; $P = 0.678$). However, *L. terrestris* was able to remove more infected-straw than *A. caliginosa.* In addition, *L. terrestris* significantly reduced the presence of *Fusarium* biomass and DON. *A. caliginosa* appeared not to play a substantial part in the removal of *Fusarium* biomass and DON. The

authors' field results are in agreement with their laboratory observations, in which *L. terrestris* displayed the potential to reduce both the pathogenic fungi and DON [10, 11].

Discussion

All of the mycotoxin studies with earthworm conducted to date have considered DON and have shown that lethality does not occur at high concentrations (range from 123 to 146 mg DON/kg soil). In addition, the body weight of the earthworms appears to increase when exposed to *Fusarium*-infected straw and DON. It has also been established that DON bioavailability occurs during fungal digestion, and the highest concentrations of DON have been found in the gut tissue, intermediate concentrations in the body wall of the earthworms. The gut content appears to contain the lowest concentrations of DON.

The removal of *Fusarium* biomass and DON degradation by *A. caliginosa* was similar when compared to microcosms without *A. caliginosa*. However, this is to be expected since the genus *Aporrectodea* is classified as an endogeic (upper soil) species versus epigeic (surface soil and litter) species. The removal of *Fusarium* biomass and DON degradation was not contributed to *A. caliginosa* feeding; therefore, microbial activity may have been responsible for the percent of *Fusarium* biomass and DON that was not present at the end of the experiment. Therefore, if *A. caliginosa* contributes to the removal of pathogenic fungi, it will likely occur below the soil surface rather than at the soil surface. *Lumbricus terrestris* is an anecic species known for deep-burrowing behavior; however, *L. terrestris* feeding usually occurs around surface litter. This may explain the increased removal of *Fusarium*-infected straw by *L. terrestris* versus *A. caliginosa* on the soil surface [14, 15].

It is unclear if the increase in body weight in the study conducted by Oldenburg et al. (2008) was a result of increased feeding behavior involving food preference for *Fusarium*-infected straw or if it was a result of mycotoxin exposure (i.e., DON) [10]. In addition, the uptake of DON by *L. terrestris* is difficult to estimate because the removal of DON (i.e., 88%) was reported in soil microcosms with no earthworms. Thus removal may have been due to soil microbial activity. The DON concentrations found in the initial *Fusarium* biomass were not compared to estimated environmental concentrations (EEC) or the likelihood of occurrence. It is assumed that the DON concentrations are high; however, the increased magnitude relative the EEC was not discussed. Currently there are two methods in which the EEC of the toxin/xenobiotic of interest may be addressed in earthworm ecological studies: (1) field monitoring for a particular geographic location (2) computational modeling. In the next chapter computational modeling was used to forecast the EEC of FB_1 in Iowa corn fields.

Many ecotoxicological studies using earthworms report changes in body weight as a toxicological endpoint. However, many researchers only collect the initial and final total biomass and do not report individual weights. Reporting the change in total biomass versus individual weights reduces the statistical power to determine significant variation within and among treatments. This review of the current earthworm mycotoxin literature indicates that the toxicological effects from DON are negligible on earthworms, even at the highest doses used to date. Also laboratory results coincide with field results [13]. However, the reproductive and developmental effects of DON exposure on earthworms remain unknown.

Fumonisin - Another Mycotoxin of Ecological Concern

Fusarium verticillioides (previously known as *Fusarium moniliforme*) is the main producer of the mycotoxin fumonisin B_1 (FB_1) [16]. In the Midwest *F. verticillioides* is typically a common fungus found on corn plants before and after tillage practices. Therefore addressing the ecotoxicological hazard potential for FB_1 in Midwest agriculture fields versus other mycotoxins is more environmentally representative. The next chapter addresses the potential hazard of FB_1 exposure to *Eisenia fetida* earthworms in a microcosm containing artificial soil.

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CHAPTER 6

ACUTE ECOTOXICOLOGICAL STUDY OF FUMONISIN B_1 TO *EISENIA FETIDA* EARTHWORMS

Abstract

Fumonisin (FB) mycotoxins are ubiquitous in nature, infecting corn throughout the world. The state of Iowa supplies 16% of the United States' corn production, and corn serves as a major dietary component for swine (e.g., ~ 34 to 86% of ration), a combination that leads to the sustainability of Iowa's large swine enterprise. Fumonisins, which may commonly occur in grain, are suspected to have the potential for environmental cycling, which could be exacerbated by common agronomic practices such as land application of swine manure. The high volume of Iowa's corn and swine production could contribute to FB environmental exposures with unknown effects to terrestrial invertebrates.

To assess the ecotoxicological hazard potential of fumonisin B_1 (FB_1) on terrestrial invertebrates, an acute 14-d microcosm study was conducted under controlled laboratory conditions by exposing the earthworm species *Eisenia fetida* to FB_1 in an artificial soil (AS) system. Exposure concentrations were 1-, 3- and 6-fold greater than the estimated environmental concentration (EEC): 2 mg FB_1/kg AS (the EEC), 6 mg FB_1/kg AS and 12 mg FB_1/kg AS, respectively. Fumonisin B_1 was treated onto alfalfa meal, which was used as the food source. *E. fetida* survival and growth were measured in each treatment. *E. fetida* survival was 100% in all treatments. Results revealed a percent mean individual body weight increase from day 1 to day 14 for the negative control and all FB_1 treatments: 24% (negative control), 34% (1xEEC), 35% (3xEEC) and 30% (6xEEC). Relative to the negative control,

treatment 1 increased percent mean individual body weight by 9%, treatment 2 by 10% and treatment 3 by 5%. Percent mean individual body weight declined by 45% for the positive control (500 mg pendimethalin/kg AS). Considering the conservative dosimetry exceeding the EEC by a maximum of six fold, it is concluded that acute environmental exposures of FB_1 presents minimal ecotoxicological hazard potential to *E. fetida*.

Introduction

Fumonisins are mycotoxins (secondary metabolites) produced by the fungus *Fusarium verticillioides* (syn = *F. moniliforme*) and other species. *F. verticillioides* is capable of producing a series of approximately 15 fumonisin (**FB**) metabolites that differ in production rates and associated toxicity. Research has shown that fumonisin B_1 (**FB_1**) is the most prevalent metabolite of FB and is associated with the highest toxicity [1]. Toxicological effects associated with FB_1 range from porcine pulmonary edema, leukoencephalomalacia, and liver and kidney carcinogenesis in rodents. The International Agency for Research on Cancer has classified FB_1 as a Group 2B carcinogen (possibly carcinogenic to humans) [2]. Epidemiological studies have concluded an increased prevalence of human esophageal cancer and neural tube birth defects associated with dietary exposure to FB [3, 4, 5].

Fusarium verticillioides is ubiquitous in nature, predominately infecting corn (*Zea mays L.*) throughout the world and sometimes producing FB. Extensive research has been conducted to understand the production of FB in corn and its impacts on corn-based food, feed and animal production industries [6, 7, 8]. However, limited research has been conducted to understand the environmental fate of FB in soils [9, 10, 11]. Effects on

terrestrial organisms remain unknown and the majority of FB ecological studies have focused on aquatic toxicology [12, 13, 14, 15].

As corn is a primary commodity with significant FB-contamination, corn serves as the initial source of contamination for the overall exposure to FB. The World Health Organization review of FB concludes the majority of FB will be recycled into the environment and as a consequence will become spatially concentrated [1]. Reasons for FB persistence in the environment are likely due to its physicochemical properties of heat and light stability, high water solubility, low absorption, distribution, metabolism, and rapid excretion by animals [1, 16]. Therefore, FB has the potential to concentrate in agricultural soils along a contamination pathway involving corn, swine feed, swine manure and land application of manure as a fertilizer source.

Currently no information exists regarding the toxicity of FB to soil organisms which may result from FB cycling and concentration in agricultural soils. This study is the first attempt to understand the exposure and effects characterization of FB_1 to earthworms by conducting an acute earthworm ecotoxicological study (14 d) using weight gain (variation in bodyweights) of adult earthworms as the toxicological endpoint.

Materials and Methods

Test Organism and System

Eisenia fetida was selected as the test organism. The use of *E. fetida* as a reference earthworm for ecotoxicological studies has been established internationally [17, 18]. Many ecotoxicological studies use *E. fetida* due to its commercial availability and ease of culturing

in laboratory conditions. In addition, sexual maturation and reproductive rates are high relative to many other earthworm species.

Adult *E. fetida* were obtained locally (The Soil Kitchen Inc., Ankeny, IA). To obtain age-synchronized individuals (3 to 4 weeks variation), culturing systems were built using plastic containers (Rubbermaid®; 60.7 x 40.4 x 22.1 cm) with small holes in the lids and stored in the laboratory with an average temperature of 22°C ± 1°C. Earthworms were cultured in composted coco peat (Coco Tek®) to limit fungal growth during establishment and age-synchronization. *E. fetida* remained under constant darkness with the exception of feeding times. Dehydrated alfalfa meal (ARCO®, Lake Park, IA) was moistened to approximately 50% moisture and used as a food source. To establish populations, approximately 100 cocoons (egg cases) were selected from 8 different culturing systems and placed into fresh culturing media with no earthworms. As the *E. fetida* population continued to grow, earthworms were placed into new culturing containers with fresh growing media when needed to prevent cast-material intoxication.

Due to the fungal growth that was observed during probe studies using sphagnum peat, the test system followed the artificial soil (**AS**) methods describe by De Silva and van Gestel [19], which substituted sphagnum peat with composted coco peat. The artificial soil in this ecotoxicological study consisted of 70% Silica Sand F-65 Ottawa (SiO_2 99.77%; Laguna Clay Company®), 20% pure Kaolin (ACROS Organics®) and 10% composted coco peat (Coco Tek®) [19]. The coco peat exhibited neutral pH as recommended by common protocol [19] and so was used without further modification. All earthworms were acclimated into the artificial soil for 20 days before introduction to the test systems. Age-synchronized adults with a well-developed clitellum (400 to 505 mg dry weight) were used for the

ecotoxicological assay and randomly placed into 950 mL Pyrex® containers (14.2 cm x 6.3 cm; n = 10, 4 replicates for all treatments and controls) with 500 g of fresh AS adjusted to 35% (dry weight) moisture content through addition of distilled water. Test systems for the ecotoxicological study were placed in an incubator at 23°C under constant darkness for 14 days, and individual dry weights of earthworms were recorded before and after incubation.

Exposure Characterization

Conceptual Model

Swine manure application as a fertilizer is a common practice in the Midwest and is considered here the dominant source of FB_1 to which soil invertebrates may be exposed [20]. The majority of FB_1 intake by swine is eliminated intact, which was found by the elimination profile of radioactive FB_1 administered as a single intravenous dose and was similar to that observed with long-term (33-day) feeding [21, 22]. Both studies concluded that urinary elimination accounted for ≈ 0 to 2.5% FB_1 recovery of the total dietary exposure, while fecal material was the main source of FB_1 elimination (≈ 80%). The fate of FB_1 is generally considered to be environmentally stable and to cycle and concentrate [1]. Therefore, it is conservatively assumed that FB_1 occurring in swine manure will remain intact for the purposes of the following exposure estimate.

To calculate the estimated environmental concentration (**EEC**) of FB_1 to soils from swine manure applications, the maximum manure application rate was estimated using default parameters of the Iowa State University Extension Dry Manure Calculator (Table 1) [23]. Calculations are based on the mass of manure generated per day, as the majority of manure application involves broadcasting without incorporation and because feces is the major route of FB_1 excretion [20, 22]. The Iowa State University Extension Dry Manure

Calculator loading estimate for manure was based on the soil/plant nutritional needs for a corn following corn rotation.

The FB_1 dietary exposure estimate (mg FB_1/kg diet) is based on grower-finisher pigs from Delgado and Wolt (2011) [8] using a dietary ration composed of non-Bt corn and non-Bt DDGS as a high end estimate, as non-Bt corn will have higher concentrations of FB_1 as compared to Bt-corn [6]. On this basis, the predicted 90th percentile for grower-finishing pig exposure to FB_1 in diets is 7.87 mg FB_1/kg diet.

Assuming an average daily feeding intake (ADFI) of 2.16 kg [24], equation 1 is used to estimate the amount of FB_1 in the ADFI.

$$7.87\ \frac{\text{mg FB1}}{\text{kg diet}} * 2.16 \frac{\text{kg}}{\text{ADFI}} = 17.0\ \text{mg} \frac{\text{FB1}}{\text{ADFI}} \quad (eq.1)$$

Since 80% of ingested FB_1 from the ADFI is excreted in feces (13.6 mg FB_1 per day), and assuming manure production per hog of 0.175 lb/day (0.08 kg/day), the estimated FB_1 concentration in manure is approximately 170 mg FB_1/kg feces (170 g FB_1/Mg feces). Thus, based on the estimated maximum application manure rate as a source of N for corn (9.50 T/acre = 21.3 Mg/ha; Table 1), the conservatively estimated FB_1 loading to the surface 15-cm of a typical soil is 1.81 g FB_1/Mg soil (EEC ≈ 2 mg FB_1/kg soil, see Table 2).

Experiment Design and Dosing

The experimental design consisted of a positive and negative control and three treatments of FB_1. The herbicide pendimethalin (Prowl®; Supelco) was applied to the positive control treatments (500 mg/kg AS), as the effects of pendimethalin on *E. fetida* earthworms have been characterized [25, 26, 27]. Acetone was used to dissolve the

pendimethalin, and the introduction of pendimethalin to the food source (alfalfa meal, 3.0 g) was added a day before the addition of *E. fetida* to aid in the removal of acetone. The negative control was composed of the AS mixture as described above without the addition of FB_1. The FB_1 treatments consisted of three dose levels centered on the EEC (1xEEC, 3xEEC, and 6xEEC), resulting in FB_1 dosing concentrations of 2 mg FB_1/kg soil, 6 mg FB_1/kg soil, and 12 mg FB_1/kg soil. Earthworms were removed from the culturing container using nitrile gloves and cleaned with DI water, patted dry with filter paper, weighed and placed in the soil test system. Ten adult earthworms (400 to 505 mg dry weight) were randomly assigned per treatment.

After the addition of earthworms to the test systems, all FB_1 treatments were immediately spiked onto the food source (3.0 g of alfalfa meal). The spiking procedure included the addition of distilled water (1 mL) to the vials containing FB_1 and was vortexed for 1 minute for each treatment. The use of organic solvents was not used during the spiking procedure because FB_1 is highly water soluble (25 mg/mL) and weakly adsorbed to organic material (log P = 1.84). Pure FB_1 was purchased in powder form from Sigma-Aldrich.

Statistical Analysis

Statistical analyses were performed by using SAS system Version 9.3 (Cary, N.C. USA) for individual body weights of earthworms (mg) measured at the beginning and the end of the experiment (day 1 and 14). Data was normalized by log-transformation. Mean separation analysis was conducted by a *t*-test at a 0.05 alpha level.

Results

After 14-days of incubation the survival rate of the entire study was 100%. *E. fetida* for the positive control displayed lethargic behavior and some discoloration. The initial introduction of food for the positive control was not consumed entirely, as result no additional food was applied over the course of the study. Additional food was required for all other treatments and negative control. Soil avoidance behavior was not observed during the entire study.

All treatments with the exception of the positive control displayed an increase in mean individual body weights, however, significant differences of mean individual body weight gain were reported for treatment 1 (2 mg FB_1/kg AS; 1xEEC) and treatment 2 (6 mg FB_1/kg AS; 3xEEC) ($F = 155.02$, $p < 0.001$) on day 14 (Figure 1). Relative to the negative control, earthworms in treatment 1 increased in percent mean individual body weight by 9%, treatment 2 by 10% and treatment 3 by 5%. The percent mean individual body weight declined by 45% for the positive control (Figure 2).

Discussion

Other Environmental Pathways for Fumonisin Exposure

Today's growing world population requires a larger agricultural industry, resulting in amplified crop and animal production. Larger herd sizes have resulted in various methods of agricultural waste management including use of manure as a fertilizer and source of organic matter. Growth in swine production trends has driven the need for the increased construction

of earth waste storage structures (**EWSS**) at both the state (e.g., Iowa) and national level. However, the majority of manure field application occurs in the Midwest (Figure 3). Waste generated by confined animal feeding operations has resulted in concerns about the risk of groundwater and surface water contamination due to EWSS construction failure and/or spills during manure transportation to agricultural fields [28, 29, 30].

The increased popularity of conservative tillage practices could also serve as point-source for FB exposure to soils. Research has shown an increased prevalence of *Fusarium* species caused by large quantities of crop residues from conversation tillage, which serves as a long-term source of pathogenic fungi and possible mycotoxin production in soils [31, 32, 33, 34]. Earthworms in agricultural settings where conservation tillage takes place will likely experience greater exposure to mycotoxins because of their feeding ecology, which has a strong preference to fungus-infected plant material [35, 36, 37].

Importance of Estimated Environmental Concentrations (EEC)

Sometimes the dosimetry of ecotoxicological research is centered on the hazard identification generated by a range-finding test. In the present study a model was developed to assess the EEC (2 mg FB_1/kg AS) in Iowa soils. This information allowed the researcher to design conservative estimates of exposure and a worst-case scenario (i.e., 12 mg FB_1/kg AS; 6xEEC), by centering the dose at or above the EEC. Spiking the alfalfa meal with FB_1 increased the likelihood of exposure and concentration.

The use of Eisenia fetida

Eisenia fetida is a well-established test organism for ecotoxicological research, [18, 38]. Common Midwest earthworms such as *Aporrectodea tuberculate*, while providing a better ecological representative species, have a much lower likelihood of FB_1 exposure due to

their subsurface feeding ecology. *E. fetida* display surface feeding behaviors, which is suited for the experimental design with contaminated surface food.

Conclusion

Fumonisin B_1 even at a level 6-fold the EEC poses minimal risk of acute toxicity to surface-feeding earthworms. Therefore, the application rates of manure used on Iowa corn fields following corn rotation are unlikely to induce toxicological adverse effects. Due to the lack of acute toxicity of FB_1 it may be possible for earthworms to reduce pathogenic fungi from conservation tillage practices. To build upon the ecotoxicological knowledge base of this study, long-term reproductive studies using other species of earthworms with different feeding patterns (e.g. *Aporrectodea tuberculate*) should be conducted.

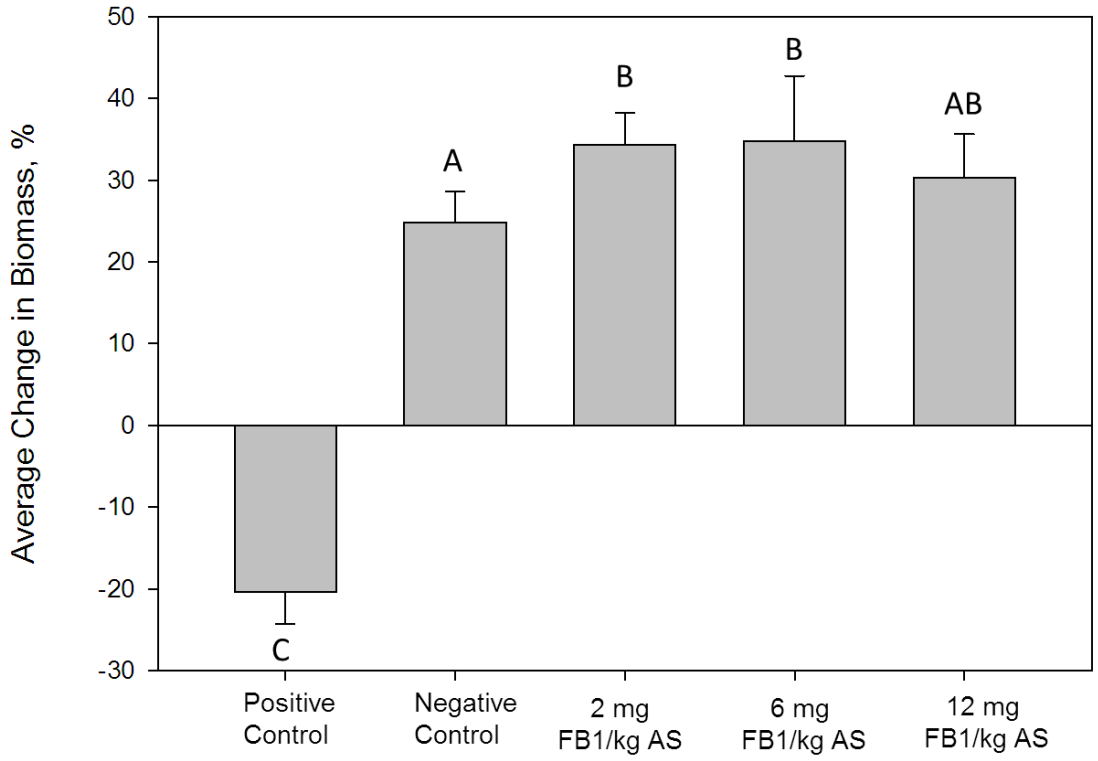

Figure 1. [1]Percent change in mean individual body weights on day 14

[1]Means with the same letter are not significantly different.

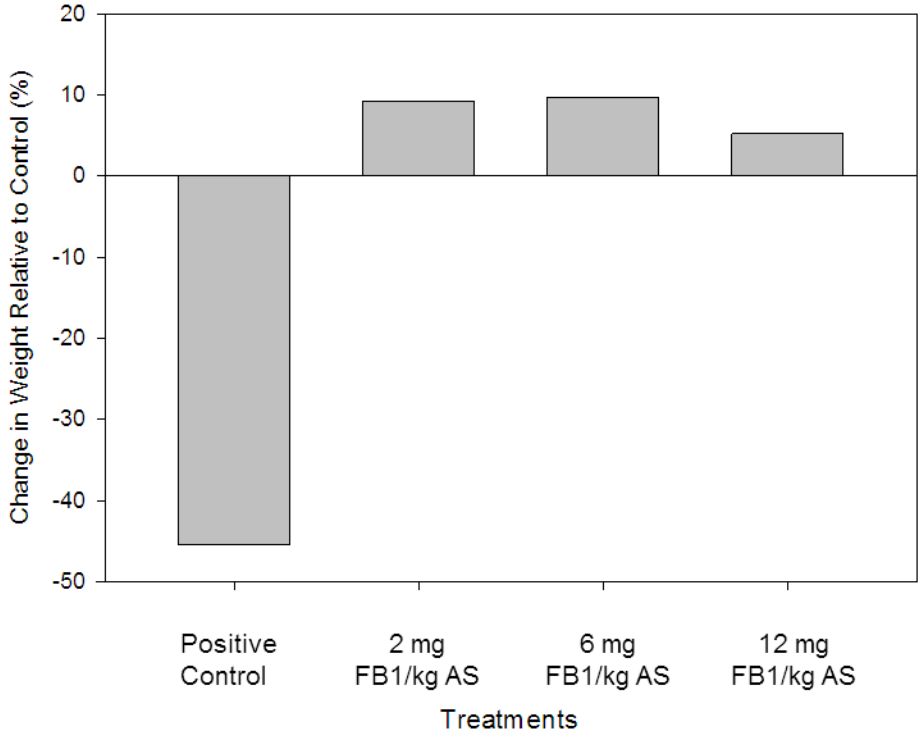

Figure 2. Percent change in mean individual body weights on day 14 relative to the negative control

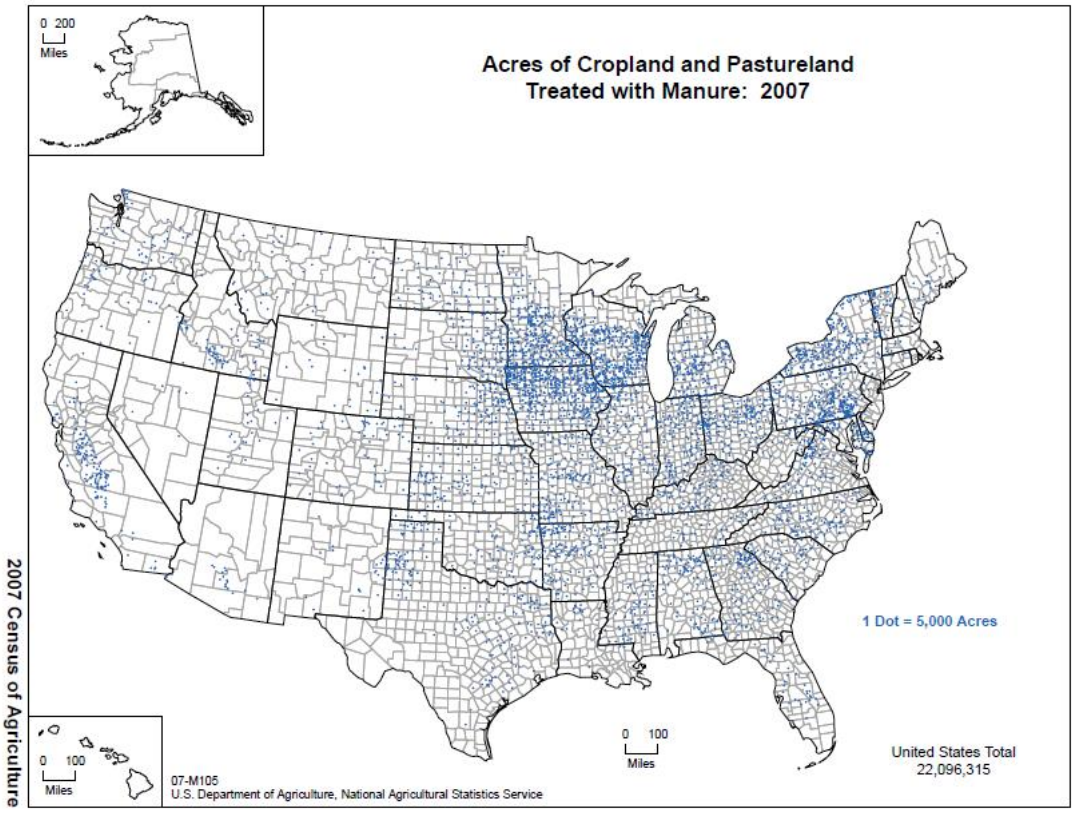

Figure 3. Acres of cropland and pastureland treated with manure during 2007 in the United States

Table 1. Iowa State University Swine Manure Calculator and model inputs to determine application rates

Total Manure Production			
Lot Avg. Capacity	200	head	
Manure production per head*	35	lbs/day	
Operating days	300	days/year	
Lbs / Head / Year	10500	lbs./hd./yr	
Manure produced	**2,100,000**	**Lbs./yr.**	1,050 T/yr.

Manure Test			
	N	P	K
Average test, lb./T	22	16	14
% available	100%	100%	100%
Lbs. available/T	22	16	14
Lbs. available/acre	**209**	**152**	**133**

DNR Manure Plan Maximum Application Rates*			
5 year county avg. corn yield	158.4	bu./acre	
plus 10%		174.24	bu./acre
N multiplier for area	1.2	lbs./bu.	
Corn nitrogen need		209.1	lbs./acre
less legume credit	0	lbs./acre	
Maximum acceptable N rate		209.1	lbs./acre
Nitrogen availability/T	22	lbs./T	
Maximum application rate	**9.50**	T/acre	

Table 2. Determination of FB_1 estimated environmental concentration (EEC) in soil from manure applications from grower-finisher pigs[1].

Input Parameter	Value	Unit	Rationale	Reference
[FB1] in Grower-Finisher Phase (A)	7.87	FB1/kg diet	90th Percentile	[8]
Average Daily Feed Intake (ADFI)	2.16	kg	Maximum	[24]
Pig Production Capacity, (PPC)	200	pigs	Small Operation	[23]
PPC Manure Generation (PPCMG)	16	kg/day	Maximum	[23]
FB_1 Percent Excretion Rate (ER)	.80	percent	Maximum	[22]
Manure Production Per Day (MPPP)	0.08	kg/day	PPCMG/PPC	
Application Rate of Manure (ARM)	21.3	Mg/ha		[23]
Manure Incorporation & Soil Bulk Density (M)	$2x10^3$	Mg soil/ha	Incorporation 15 cm depth, soil bulk density $1.33g\ cm^{-3}$	[39]

[1]Estimated Environmental Concentration (EEC) = (A x ADFI x ER x ARM) / (MPPP x M) ≈ 2 mg/kg soil

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CHAPTER 7

Conclusions

Research was conducted to estimate the exposure and effects characterization of FB_1 in nursery and grower-finisher pigs. In addition, an ecotoxicological study was conducted to assess hazard potential from FB_1 exposure to the earthworm *Eisenia fetida.*

Nursery Quantitative Dietary Exposure Assessment

The stochastic analysis generated in this study forecasts long-term FB_1 exposures ranging from 1 to 4 mg FB_1/kg diet from corn and DDGS products in the currently used diets of nursery swine. The information generated from this investigation not only provides an exposure characterization for FB_1, which could assist in the experimental design for chronic low-dose toxicological studies for nursery swine, but also provides information which could aid in the reduction of FB from swine diets.

Scenarios representative of the diet that is commonly used in the swine industry in the Midwestern USA based on acceptance of genetically engineered corn and the increased use of DDGS in swine diets predicted little potential for adverse effects from long-term exposure to swine populations in nursery facilities. However a scenario where GE corn was absent in feed predicted a high frequency of possible body weight reductions characteristic of FB_1 toxicity. Based on this current investigation, it is not likely for the swine industry to experience toxicological adverse effects from long-term FB exposure sufficient to impact productivity. Use of Bt maize in diets appears to afford adequate margins of exposure to limit adverse effects of prolonged FB_1 exposure to swine in the nurseries.

Grower-Finisher Quantitative Dietary Exposure Assessment

Semi-stochastic analysis predicted FB_1 dietary exposure ranging from 1.50 to 5.08 mg FB_1/kg diet for the mean and 2.52 to 7.87 mg FB_1/kg diet for the 90th percentile, where the chronic toxicological incipient level of concern is 1.0 mg of FB_1/kg of diet. The current lack of FB_1 toxicological data for grower-finisher pigs makes it difficult to predict the possible adverse effects in diets exceeding the LOC. To aid in the understanding of FB_1-mycotoxicosis, future studies should investigate chronic low-dose exposures. In addition, to understand the lifetime exposure (utero-to-finish) of FB_1 on health/growth, further QEA models will be required for the gestation phase.

Ecotoxicological Study

There appears to be minimal risk of acute toxicity to surface-feeding earthworms associated with conservative exposures of FB_1 (six fold of EEC) when the toxicological endpoint is assessed by change in body weight. Generation of the EEC model indicates that application rates of manure used on Iowa corn fields are unlikely to induce acute toxicological adverse effects.

APPENDIX

A. Fumonisin B_1 concentrations in paired trials of Bt and non-Bt hybrids used to estimate FB_1 in swine diets.

[1]Original data contained total fumonisin ($FB_1 + FB_2 + FB_3$) which was converted to fumonisin FB_1 concentrations (fumonisin B_1 = total fumonisin/1.4142), R2 = 0.9968.

[2]Data quantified as fumonisin B_1.

Location - Year	FB_1 from non-Bt corn (mg FB_1/kg corn)	FB_1 from Bt-corn (mg FB_1/kg corn)	Reference
Story County, IA - 1995	1.20	2.43	[2]Munkvold et al.,1999
	1.20	2.23	
	8.81	6.71	
	8.81	3.04	
Story County, IA - 1996	0.51	0.46	
	2.03	1.81	
	1.05	1.06	
	0.64	0.34	
	2.39	1.09	
	8.72	2.63	
	3.15	3.70	
	5.49	1.39	
Boone County, IA - 1997	4.88	4.36	
	2.82	4.27	

	5.65	9.00	
	6.07	6.13	
	13.85	0.64	
	6.39	0.48	
	17.39	2.28	
	11.03	8.83	
	7.87	7.42	
	10.11	6.09	
	14.40	0.97	
	10.95	1.17	
	19.31	6.36	
	16.01	10.15	
	9.85	13.43	
	12.84	12.25	
	23.51	1.70	
	15.89	3.95	
	16.02	2.11	
	10.96	10.00	
	8.75	5.82	
	5.52	8.22	
	18.82	1.11	
	15.81	1.46	
Story County, IA - 1997	6.36	0.49	[2]Munkvold et al., 2000
	2.91	4.05	

	5.97	5.90	
	13.76	0.72	
	11.13	1.14	
	10.95	8.67	
	10.05	5.94	
	14.33	0.98	
Story County IA - 1998	8.17	1.31	
	3.33	1.01	
	4.34	0.91	
	21.78	1.82	
	10.69	7.46	
	22.78	2.62	
Story County, IA - 1999	1.13	9.55	[1]Munkvold et al., 2000
Peoria, IL - 1996	1.98	0.57	[1]Dowd 2000
Kilborurne, IL - 1996	0.63	0.37	
1997	0.78	0.02	
	1.84	2.47	
	0.99	0.03	
1998	0.14	0.01	
	0.99	0.28	
	0.14	0.04	
1996	6.22	1.41	

	0.25	0.25	
1997	0.92	0.11	
	3.39	1.98	
1998	0.16	0.02	
	0.04	0.18	
Manito, IL – 1998	0.28	0.02	[1]Dowd 2001
Easton, IL - 1998	0.76	0.52	
	0.76	0.26	
	0.76	0.42	
Kilborurne, IL – 1999	0.35	0.11	
Upper Rhine Valley and Barvaria, Germany - 1999	0.02	0.03	[1]Magg et al., 2002
	0.07	0.07	
	0.22	0.07	
	0.15	0.07	
[2]Spain - 1999	3.01	0.55	
	9.03	0.60	
[2]France, - 1999	0.89	0.27	
	0.57	0.14	
	3.39	0.31	
Urbana & Monmouth, IL – 2001 & 2002	6.90	5.47	[1]Clements et al., 2003
	5.23	5.18	
	6.36	3.99	
	3.91	1.77	

	2.48	2.15	
	2.04	1.51	
Cukurova, Turkey - 2001	12.37	1.77	[1]Tatli et al., 2004
	11.03	1.84	
2002	16.75	0.78	
	12.70	0.63	
Imperial, NE - 2002	4.53	0.99	[1]Hammond et al., 2004
Lexington, NE	4.88	1.63	
Kearny, NE	3.75	0.64	
Hampton, NE	4.24	1.41	
Grand Island, NE	5.23	1.13	
Ogden, IA	0.35	0.35	
Ogden, IA	0.64	0.35	
Jasper, IA	1.77	1.13	
Storm Lake, IA	0.35	0.49	
Ahrens, IA	0.49	0.42	
Bonnichsen, IA	0.49	0.35	
Volga, IA	0.49	0.35	
Wall, IA	0.57	0.35	
Centralia, KS	0.42	0.49	
Onaga, KS	0.42	0.35	
Onaga, KS	1.27	0.57	
Brookings, SD	0.35	0.42	

Clinton, IL	0.35	0.35	
Clinton, IL	1.84	1.20	
Clinton, IL	1.84	2.69	
Clinton, IL	0.85	0.42	
Clinton, IL	1.48	0.35	
Clinton, IL	1.48	0.99	
Clinton, IL	0.78	0.71	
Mount Carmel, IL	0.49	0.35	
Mount Carmel, IL	1.06	0.35	
Jacob, IL	2.83	1.48	
Jacob, IL	0.35	3.61	
Florida, IL	11.17	0.35	
Staunton, IL	10.89	1.41	
Staunton, IL	3.32	6.36	
Stonington, IL	3.18	0.57	
Stonington, IL	6.51	1.20	
Stonington, IL	8.41	3.04	
Stonington, IL	5.73	1.48	
Princeton, IN	1.34	0.57	
Princeton, IN	3.54	0.49	
Northern, IN	0.35	0.35	
Ashville, OH	0.64	0.35	
Ashville, OH	2.26	0.35	

Ashville, OH	0.35	0.35	
Lockbourne, OH	0.35	0.42	
Lockbourne, OH	0.35	0.35	
York, PA	0.99	0.71	
Milton, PA	0.99	0.85	
Milton, PA	3.82	0.57	
Salisbury, MD	1.56	0.35	
Galena, MD	0.42	0.35	
Hampstead, MD	0.35	0.64	
Lexington, SC	7.35	10.11	
Lexington, SC	0.71	0.35	
Lexington, SC	3.04	5.16	
Elk Point, SD	3.46	0.85	
Oklahoma	0.42	0.57	
Alabama	29.77	15.84	
Alabama	3.46	0.49	
Texas	0.34	0.35	
Texas	0.07	0.57	
Georgia	54.45	22.49	
Tennesse	28.28	2.55	
Nebraska	2.76	0.28	
Kansas	1.56	0.35	
Kansas	1.06	0.78	
Missouri	1.98	0.92	

Missouri	3.25	0.42	
Missouri	1.27	0.14	
Iowa	1.63	0.28	
Illinois	26.09	1.63	
Illinois	1.70	0.78	
Brady, NE - 2002	1.98	0.49	
Brady, NE	4.38	1.84	
Monroe, NE	1.84	0.49	
Plainview, NE	0.35	4.88	
Spalding, NE	4.60	1.63	
Spalding, NE	3.89	0.14	
Arlington, IA	0.78	3.32	
Arlington, IA	0.64	0.21	
Deon, IA	3.04	0.71	
Deon, IA	5.52	5.59	
Donnellson, IA	0.14	1.63	
Lohrville, IA	10.82	4.95	
Lohrville, IA	8.77	2.19	
Manning, IA	3.04	2.47	
Marble Rock, IA	0.85	0.14	
Marble Rock, IA	3.11	0.85	
Marshalltown, IA	7.78	5.16	

Mount Vernon, IA	5.44	0.21	
Norway, IA	1.34	0.64	
Norway, IA	2.90	0.28	
Ogden, IA	17.89	5.30	
Oskaloosa, IA	1.56	0.14	
Orange City, IA	7.00	6.01	
Orange City, IA	14.35	11.10	
Primghar, IA	2.76	3.96	
Primghar, IA	7.28	7.57	
Shellrock, IA	1.27	1.91	
Sloan, IA	5.66	3.39	
Somers, IA	3.39	0.99	
Somers, IA	3.39	4.31	
Somers, IA	8.84	4.31	
Spencer, IA	6.22	5.23	
Spencer, IA	4.95	0.49	
Storm Lake, IA	18.60	22.06	
Storm Lake, IA	19.16	12.87	
Storm Lake, IA	12.45	2.97	
Thorman, IA	1.41	2.90	
Tripoli, IA	4.88	2.83	
Westside, IA	18.03	2.19	
Washingtion, IA	1.98	1.98	

Woodborg, IA	6.29	5.73	
Woodborg, IA	6.51	3.54	
Roshman, TX	4.17	2.12	
Roshman, TX	6.79	2.40	
Shelby, MS	5.02	0.42	
Shelby, MS	3.68	1.13	
Shelby, MS	7.21	2.55	
Shelby, MS	3.04	4.81	
Shelby, MS	3.82	0.35	
Shelby, MS	5.02	2.90	
Shelby, MS	0.78	1.98	
Shelby, MS	0.85	0.64	
Shelby, MS	7.57	1.06	
Shelby, MS	3.89	0.28	
Shelby, MS	1.20	0.28	
Shelby, MS	4.45	3.04	
Cheneyville, LA	1.34	0.64	
Cheneyville, LA	2.47	0.21	
Cheneyville, LA	1.20	1.70	
Union, TN	2.55	0.14	
Union, TN	1.70	3.75	
Union, TN	1.63	0.42	
Leitchfield, KY	0.35	0.21	
Vine Grove, KY	2.19	1.20	

Adrain, MN	0.71	2.12	
Appleton, MN	0.92	0.28	
Brewster, MN	0.07	0.49	
Clarkfield, MN	2.90	2.26	
Fairmount, MN	1.06	0.14	
Madison, MN	0.49	0.28	
Clear Lake, SD	0.49	0.21	
Bob Starke, SD	0.99	1.84	
Carrollton, MO	1.20	0.21	
Carrollton, MO	0.99	0.35	
Carrollton, MO	2.33	0.21	
Aledo, IL	6.79	0.14	
Aledo, IL	5.59	0.07	
Alexis, IL	1.48	0.14	
Alexis, IL	2.12	0.42	
Alhambra, IL	0.14	0.28	
Assumption, IL	0.64	1.84	
Dundas, IL	0.14	0.21	
Dundas, IL	0.57	0.49	
Flanigan, IL	0.92	0.42	
Flanigan, IL	0.85	0.35	
Flanigan, IL	2.97	0.07	
Flanigan, IL	2.97	0.64	
Flanigan, IL	0.85	0.28	

Flanigan, IL	2.47	0.14	
Flanigan, IL	1.56	6.72	
Flanigan, IL	1.98	3.61	
Flanigan, IL	9.62	1.20	
Girard, IL	1.41	0.42	
Girard, IL	3.04	2.05	
Gridley, IL	2.83	0.21	
Gridley, IL	0.64	0.42	
Monmouth, IL	0.42	1.56	
Monmouth, IL	1.34	0.14	
Monmouth, IL	1.20	2.97	
Monmouth, IL	1.91	0.21	
Monmouth, IL	10.96	7.57	
Monmouth, IL	1.41	1.98	
Monmouth, IL	0.64	8.63	
Morris, IL	0.28	0.57	
Morris, IL	1.77	0.14	
Morris, IL	0.49	1.06	
New Berllin, IL	0.42	0.14	
Princeton, IL	2.97	0.14	
Princeton, IL	3.75	0.28	
Princeton, IL	1.34	3.54	
Raymond, IL	2.76	0.14	
Seaton, IL	0.92	0.71	

Seaton, IL	2.55	0.14	
Wyoming, IL	1.13	0.14	
Yates City, IL	0.64	0.14	
Yates City, IL	1.34	0.14	
Berne, IN	1.13	0.14	
Berne, IN	0.85	0.21	
Evansville, IN	6.01	3.04	
Monticello, IN	0.92	0.35	
Monticello, IN	0.85	0.21	
Rochester, IN	0.92	0.42	
Rochester, IN	1.48	0.14	
West Lafayette, IN	2.05	0.14	
West Lafayette, IN	2.05	0.14	
Moline, MI	3.96	2.69	
Moline, MI	0.78	0.14	
Moline, MI	2.90	4.17	
Sand Lake, MI	5.44	1.70	
Raymond, MS	3.75	2.90	
Washingtion Co., NC	4.81	2.05	
Washingtion Co., NC	3.11	4.10	
Spalding Co., GA	1.77	1.13	

Colombia, MO	0.99	1.91	
Jerseyville, IL	5.59	1.20	
Massac Co., IL	28.28	2.69	
Pope Co., IL	11.95	1.84	
Garden City, KS	7.99	6.36	
Beaconfield, IA - 2002	1.34	3.46	
Beaconfield, IA	3.54	1.98	
Danbury, IA	3.82	4.53	
Danbury, IA	2.05	4.10	
Danbury, IA	5.02	4.45	
Ogden, IA	5.44	1.20	
Ogden, IA	3.18	2.97	
Ogden, IA	6.29	0.92	
Persia, IA	6.72	4.24	
Persia, IA	3.11	3.39	
Persia, IA	8.63	11.31	
Sloan, IA	2.69	0.49	
Sloan, IA	11.10	12.37	
Elliott, IA	0.21	0.14	
Elliott, IA	1.13	0.35	
Elliott, IA	0.92	0.28	
Perry, IA	1.63	2.76	

Perry, IA	2.40	4.88	
Pacific Junction, IA	11.88	7.07	
Pacific Junction, IA	18.17	3.04	
Pacific Junction, IA	6.08	12.59	
Westside, IA	9.55	5.16	
Westside, IA	7.14	10.39	
Westside, IA	15.91	8.20	
Lohrville, IA	8.77	3.11	
Lohrville, IA	5.66	2.69	
Lohrville, IA	3.25	3.04	
Seelow,Germany - 2001	3.38	0.95	[1]Papst et al., 2005
Fresing, Germany	0.08	0.08	
Heilbronn, Germany	3.71	0.22	
	0.08	0.08	
	3.34	0.44	
	0.08	0.08	
	3.30	0.08	
	0.05	0.08	
Fontezuela, Argentina - 2000	12.42	9.06	[2]de la Campa et al., 2005
Salto, Argentina	9.79	4.42	

Bragado, Argentina	4.15	0.74	
Ocampo, Argentina - 2001	7.36	1.24	
Roja, Argentina	0.30	0.13	
Bragado, Argentina	4.81	0.84	
Pinto, Argentina	3.39	0.47	
Philippines: 2001			
	1.85	1.25	
Cauayan, Isabela Bukidnon, Kibawe	0.43	0.49	
Philippines: 2002			
	0.82	0.28	
Cauayan, Isabela Bukidnon, Kibawe	0.21	0.21	
Clay County, SD - 2003	2.12	1.17	[1]Catangui & Berg 2006
	1.06	0.85	
	0.07	1.41	
	0.07	2.83	
	1.91	0.99	
	2.12	0.99	
	1.06	0.64	

	2.19	0.14	
	1.98	2.55	
	1.34	1.91	
	4.17	2.86	
	3.57	0.57	
	1.84	3.75	
	1.27	0.85	
	1.91	0.64	
	1.63	2.55	
	1.13	1.03	
	0.00	1.13	
	1.70	0.21	
	0.57	0.14	
	1.13	0.85	
	0.14	0.42	
	1.70	0.49	
	0.57	0.71	
Clay County, SD - 2004	1.20	0.14	
	0.14	0.14	
	0.14	0.14	
	0.28	0.14	
	0.14	0.21	
	1.84	0.14	
	0.42	0.21	

	0.14	0.14	
	0.14	0.14	
	1.84	0.14	
	0.42	0.14	
	0.14	0.14	
	0.14	0.14	
	0.14	0.14	
	0.21	0.14	
	0.14	2.83	
	0.14	0.14	
	0.14	0.42	
	0.21	0.14	
	0.14	0.14	